Development of a Supplement for CHO Cell Culture Serum-free Media by the Fractionation of Peptide mixtures using Nanofiltration

by

Sonja Bissegger

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I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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Abstract

The objective of this work was the investigation of nanofiltration as a potential avenue to fractionate protein hydrolysates and produce protein hydrolysate fractions with stimulating bioactivity for the development of a supplement for a serum-free media.

Mammalian cell culture is widely used for the production of therapeutic proteins such as antibodies, interleukins, and vaccines because of the ability of mammalian cells to glycosylate proteins. A complex media with the addition of serum is often required to meet the requirements of the cells. Although serum is a supplement that provides different proteins such as growth factors and hormones, serum has several disadvantages such as high cost, difficulty of downstream processing due to its high protein content and the possibility of microbiological contamination. Protein hydrolysates from plant, animal, or yeast cells contain a complex mixture of peptides and amino acids and have been shown to enhance growth of certain mammalian cell lines cultured in serum-free media.

To fractionate peptide mixtures, nanofiltration was investigated in this study. Nanofiltration is a pressure driven membrane separation process based on size and charge. The investigation of pH and NaCl on the filtration performance for two different nanofiltration membranes (HL membrane and G-10 membrane) was achieved using a 2^4 factorial design. The total peptide concentration, the antioxidant activity, and organic and inorganic content were analyzed in the permeate and retentate fraction. The fractions were also tested for their enhanced growth ability and the specific β-interferon productivity with CHO cells. Furthermore the retentate and permeate fractions were analyzed by reversed phase-HPLC to characterize the peptide and free amino acid distribution profile.

Through the factorial design, the membrane type was shown to have a significant effect on the filtration performance for both yeast extract and Primatone. A significant difference, but similar for both feed sources, was observed for the total peptide transmission with around 10% for the HL membrane and around 30% for the G-10 membrane. The average permeate flux was significantly lower for the G-10 membrane although the G-10 membrane is a loose nanofiltration membrane with a reported 2500 Da MWCO compared to the HL membrane with a reported 300-500 Da MWCO. The total peptide transmission, organic and inorganic content of the fractions for the two feed sources and membrane type were affected differently according to pH and NaCl addition. These results indicate that the two feed sources are of different composition and that nanofiltration is a possible method to fractionate peptides.

The bioactivity of the nanofiltration fractions was tested as a nutrient additive to a serum-free media in CHO cells. It was shown that the productivity is not always related to the cell density, as the highest overall specific β–interferon productivity was achieved for low cell density similar to the hydrolysate free negative control. Furthermore, the retentate fraction of yeast extract separated with the G-10 membrane at a pH of 8 resulted in the highest cell density. According to these results, nanofiltration is a promising method for the enrichment of protein hydrolysates as a supplement for serum in cell culture.

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Chapter 1: Introduction

Mammalian cells are widely used for the production of therapeutic proteins because of their ability to glycosylate proteins which is a post-translational modification of proteins and enhances the bioactivity of those proteins. The cultivation of mammalian cells is however still a challenge. The addition of serum to the media is often required to add all necessary components for the growth of the cells. Serum has several disadvantages such as the possible microbiological contamination, difficulty in downstream processing caused by the high protein content present in serum, high cost, and batch to batch variations.

The absence of serum, however often leads to poor growth behaviour and productivity. Therefore a media enhanced with supplements is required to assure the nutrient needs of the cells. It has been shown in the literature that the addition of protein hydrolysates from different sources such as soy, wheat gluten, lactalbumin, or yeast stimulates the growth of certain mammalian cell lines in absence of serum. The fractionation by ultrafiltration and size exclusion chromatography of those protein hydrolysates has also been investigated and showed promising results in enriching their nutritional value when used in media for mammalian cell culture.

As peptides are similar in size, but different in their amino acid distribution, ultrafiltration and size exclusion chromatography are not the most appropriate methods to separate them, as both processes are based on size only. Nanofiltration on the other hand separates small molecules between 100 and 1000 Da according to size and charge. Peptides are charged molecules containing neutral, acidic, and basic amino acids which leads to a different charge at a different pH. Therefore nanofiltration could be a possible method to separate peptides with a positive charge from peptides that have a negative or neutral charge.

The objective of this thesis is the investigation of a possible supplement for a serum-free media in mammalian cell culture obtained by the fractionation of yeast extract and Primatone using nanofiltration.

Chapter 2: Literature review

Current research releated to this thesis and some additional background information are presented in this section. Mammalian cell culture with the required complex cell culture media together with the work on serum-free media with protein hydrolysates as a possible supplement are first presented. The methods for the fractionation of peptides and their analysis are also summarized.

2.1 Mammalian Cell culture

Mammalian cell culture is widely used for the production of therapeutic proteins such as antibodies, interleukins, and vaccines because of their ability to glycolysate proteins. Glycosylation is the post-translational modification of proteins by polysaccharides that enhances their biological activity. The growth of mammalian cells and the expression of therapeutic proteins in mammalian cells is a challenge. This is in part due to a complex media needed to maintain the growth and obtain a high protein expression level.

Chinese hamster ovary (CHO) cells are important in the expression of a variety of recombinant proteins in the biotechnology industry. CHO cells are used to produce important therapeutic proteins such as macrophage-colony stimulating factor (M-CSF), erythropoietin (EPO), thyroid stimulating hormone receptors (TSHR), and β-interferon [Liu and Chen, 2007, Restelli et al., 2005, Stiens et al., 2000, Rodriguez et al., 2005]. CHO cells were first established in 1957 by a biopsy of an ovary of a chinese hamster. Since then different cell lines have been created to be able to grow the cells in either adherent conditions (dependent on a surface) or in suspension [Horst, 2006].

2.2 Mammalian cell culture media

Media composition is the most significant factor in cell maintenance, as the requirements of each cell line is different and dependent on a complex mixture of supplements. Media for mammalian cell culture is complex and consists of a basal media and supplements such as growth factors, mitogens, hormones, attachement factors for anchorage dependent cell lines and cytokines. A basal medium contains a carbohydrate source as energy (mostly glucose), salts, vitamins, amino acids, and in more complex basal media, lipids [Darling and Morgan, 1994]. Table 2.1 and 2.2 list the components of two common basal media, RPMI, a more basic medium and Ham's F12, a more elaborate medium. Both media contain mostly the same basic components but present in different concentrations, and the more elaborate media contains trace elements and lipids. For both media a proper supplementation is necessary. This is often achieved with the addition of serum or other undefined supplements.

The choice of a medium for a specific cell line is often empirical and arbitrary. For example, the medium for other cell lines grown in the lab or employed by other authors described in publications can be selected. The type of media has a more significant impact on the growth behaviour of primary cell culture, specialized cell types, or when growth is conducted in serum-free media [Masters, 2000].

Table 2.1 Inorganic salts and amino acids contained in two basal media. RPMI 1640 and Ham's F12 [Adapted from Darling and Morgan, 1994]

	RPMI 1640	**Ham's F12**
	mg/mL	**mg/mL**
Inorganic salts		
$CaCl_2$*$2H_2O$		44.1
$Ca(NO_3)_2$ * $4H_2O$	100	
KCl	400	223.7
$MgSO_4$ * $7H_2O$	100	147.8
NaCl	6000	7600
$NaHCO_3$	2000	1176
Na_2HPO_4	800	142
Amino acids		
Alanine		8.91
L-Arginine	200	
L-Arginine-HCl		210.7
L-Asparagine	50	15.01
L-Aspartic acid	20	13.31
L-Cysteine-HCl		31.53
L-Cysteine	50	
L-Glutamic acid	20	14.71
L-Glutamine	300	146.2
Glycine	10	7.51
L-Histindine	15	
L-Histidine-HCl * H_2O		20.96
L-Hydroxyproline	20	
L-Isoleucine	50	3.94
L-Leucine	50	13.12
L-Lysine-HCl	40	36.53
L-Methionine	15	4.48
L-Phenylalanine	15	4.96
L-Proline	20	34.54
L-Serine	30	10.51
L-Threonine	20	11.91
L-Tryptophan	5	2.04
L-Tyrosine	20	5.4
L-Valine	20	11.72

Table 2.2 Vitamins / cofactors and other compounds contained in two basal media. RPMI 1640 and Ham's F12 [Adapted from Darling and Morgan, 1994]

	RPMI 1640	**Ham's F12**
	mg/mL	**mg/mL**
Other Compounds		
Glutathione	1	
D-glucose	2000	1802
Phenol Red	5	10
Pyruvate-Na		110
Vitamins / cofactors		
Biotin	0.2	0.0073
Choline chloride	3	13.96
Folic acid	1	1.32
i-Inositol	35	18.02
Nicotinamide	1	0.037
p-Aminobenzoic acid	1	
Pantothenate-Ca	0.25	0.48
Pyridoxine-HCl	1	0.062
Riboflavin	0.2	0.038
Thiamine-HCl	1	0.337
Vitamin B_{12}	0.005	1.36
Trace elements		
$CuSO_4 * 5H_2O$		2.5
$FeSO_4 * 7H_2O$		834
$ZnSO_4 * 7H_2O$		863
Additional components		
Hypoxanthine		4.08
Linoleate-Me		0.088
Lipoic acid		0.206
Putrescine-HCl		0.161
Thymidine		0.727

Most cell lines require serum as a supplement for a high growth rate and production of the desired therapeutic proteins. It has been demonstrated that serum provides essential nutrients, attachement factors for anchorage dependent cells, and detoxificants which are mostly enzymes that decrease the toxicity of harmful substances to the cells [Darling and Morgan, 1994].

But serum has several drawbacks such as:

a) Being undefined supplements;
b) Having batch to batch variations;
c) Having possible contamination with microorganisms;
d) Increasing difficulty in downstream processing because of the high protein content;
e) Increasing possibility of changing cell line phenotypes.

For the approval of a pharmaceutical product on the market, the therapeutic protein needs to be fully characterized, and the food and drug administration (FDA) suggests that cell cultures with serum as a supplement should be avoided [Food and Drug Administration, 1993]. Therefore a chemically defined media is sought, which means that all components are of known concentration, nature, and there are no variations between different batches of the ingredients or media.

Considering all the drawbacks associated with serum, it is advantageous to develop a serum-free media, especially for cell cultures and the expression of therapeutic proteins for humans. The development of a serum-free media is however a challenge as not all of the beneficial components in serum have yet been characterized.

2.2.1 Serum-free Media

An ideal serum-free media should include a mixture of supplements which are specific to a given cell line to achieve a high growth rate and productivity and is completely or partly defined and inexpensive. The development of a serum-free medium for each cell line can be labour and cost intensive, as many additives and different combination of supplements have to be tested for their performance [Spier et al., 1991]. In a chemically defined media all components are known and the concentration of each supplement can be adjusted according to

the cell line. Bioprocessing industries desire components that are of animal-free origin, therefore new sources of supplements that have serum-like functions are required. However, there are still components from animal origin such as the two amino acids cysteine and methionine isolated from animal hair, but are also available in synthetic form.

When developing a serum-free media, the selection of the supplements should be done carefully as serum also contains different lipid forms such as cholesterol, phosholipids, triglycerides, fatty acids, fat soluble vitamins, and esterified forms of these lipids which are essential for the metabolism of the cell. Specifically in serum-free media, the lipid supplements are important as the fatty acids influence the physical properties and the fluidity of the cell membrane [Masters, 2000]. Albumins are used as lipid carriers, but also show a protective funtion because of their ability to bind heavy metals, detergents, and endotoxins [Spier et al., 1991].

Once a serum-free media is established, the next step will be to adapt the cell line to these new conditions.

There are several possibilities to adapt a cell line to serum-free conditions. The easiest method consists of gradually decreasing the serum concentration until a concentration of zero is obtained. This usually results in a very slow growth rate and also a decreased productivity. The addition of various supplements summarized in Table 2.3 is another possibility to create a medium that is chemically defined and meets the requirements of the cells. This is often incorporated with intensive cost and labour. The addition of protein hydrolysates as a supplement also has been reported to yield normal growth behaviour and sometimes an even higher productivity. Spier et al (1991) described the growth of hybridoma cells in serum-free conditions from the fusion step, avoiding the development of a complex serum-free media [Spier et al., 1991]. For a few specific cell lines, commercial serum-free media are available. Often the media is expensive compared to a media developed in the laboratory, but usually result in good productivity and cell growth.

Table 2.3 Potential additives for serum-free media [Adapted from Spier et al., 1991]

Carrier Proteins and Peptides	**Growth Factors and Hormones**	**Improvement of basic media**
Albumins	PDGF	*Vitamins:*
Lipoproteins	EGF	Alphatocopherol
Transferrin	bFGF	Biotin
Glycyl-histidyl-lysine	Interleukin-2 and 6	*Inorganic Salts:*
	IGF	Mg^{2+} Ca^{2+} Zn^{2+} SeO_3^{2-}
	Insulin	*Redox Potential Improvement:*
	Hydrocortison	Ascorbic acid
		Glutathion
		β-Mercaptoethanol
		α-Thioglycerol
Attachement Factors	**Protease Inhibitors**	**Lipids, steroids, fatty acids, and precursors**
Fibronectin	SBTI	Phospholipids
Fetuin	SPTI	Cholesterol
Laminin	α-Antitrypsin	Oleic acid
Fibrinogen	Aprotinin	Enanolamine

2.2.1.1 Serum substituted by protein hydrolysates

Several authors investigated the addition of protein hydrolysates as a supplement to replace serum for the growth of various cell lines. A number of studies indicate that bioactive peptides obtained from protein hydrolysates of animal, yeast, or plant sources could be the key for an increased productivity and cell density of mammalian cells cultured in serum-free media. In the following paragraphs a selection of studies on the development of a media supplemented by protein hydrolysates in different cell lines is summarized.

Microbiological media is often supplemented by yeast extract as it contains a mixture of proteins, peptides, lipids, nucleotides, vitamins, and minerals [Chae et al, 2001]. Yeast extract also has the added benefit of being inexpensive. In the brewing industry, yeast cells are a waste product and are commonly used for animal nutrition. Yeast extract is produced by autolysis or

hydrolysis of yeast cells. A derived product of yeast extract, yeastolate, the water-soluble part of autolyzed yeast, has been investigated for a number of cell lines by different authors.

Mendonça et al. (2007) investigated the effectiveness of adding peptides isolated from yeastolate by ultrafiltration with a 30kDa membrane for the insect cell line Sf-9. The cell line was cultured in a basal media containing 10% serum to which the peptides were added. To obtain even smaller fractions, the ultrafiltered fractions were separated by size exclusion chromatography (SEC) with a 700 Da exclusion limit. Inoculated at a concentration of $3 \cdot 10^5$ cells/mL, 2% w/v crude hydrolysate, 5% w/v ultrafiltration or SEC fraction was added and the maximum cell density, growth rate, and the time to reach stationary phase over a 10 day period were measured. The addition of the crude yeastolate yielded the highest enhancing effect, with 62% higher maximum cell density compared to the control with no hydrolysate addition. The permeate of the 30kDa ultrafiltration resulted in a 60% enhancement of the maximum cell density. The SEC fractions were growth stimulating as well, but resulted in a lower enhancement than for the permeate fraction.

In contrast to Mendonça et al. (2007), Sung et al. (2004) investigated the effect of yeastolate on CHO cells in serum-free media. Yeastolate, used as an additive in serum-free media at a concentration of 0.5% w/v for CHO cells, resulted in a 50% higher maximum viable cell concentration when compared to the situation with serum-free media without additives, but was 50% lower than when serum was present in the media. Although, the specific growth rate and the maximum viable cell concentration were lower for the yeastolate supplemented media, the productivity of human thrombopoietin (hTPO) was 2.8 fold higher and the length of the stationary phase was also longer when compared to the serum containing conditions.

Protein hydrolysates from plant sources, such as soy and wheat gluten, are used as food supplements and contain all different kinds of amino acids, but also contain a high percentage of the amino acid glutamic acid or glutamine respectively [Okezie and Bello, 1988, Halford et al., 1992]. Rapeseed hydrolysate on the other hand is known to have a well-balanced amino acid distribution [Farges-Haddani et al., 2006]. Therefore protein sources from plant origin have been investigated as supplements of serum-free media for mammalian cell culture.

Sung et al. (2004) investigated the addition of soy and wheat gluten hydrolysates to CHO cell cultures. Similar to the addition of yeastolate, the addition of soy and wheat gluten hydrolysates to serum-free media did not significantly increase the cell viability. In contrast, the productivity of hTPO (human thrombopoietin) showed a significant increase, 30% compared to the cultures without supplements, and was only about 10% lower than in cultures containing serum.

Franek et al. (2000) investigated a method to decrease the non-protein components (ballast nonpeptide substances or colour) contained in protein hydrolysates by a hexane precipitation since these components may affect negatively the biological activity of the hydrolysates. Different fractions were obtained after an initial 30 kDa ultrafiltration step and two subsequent SEC steps for the soy protein hydrolysate and one subsequent SEC step for the wheat gluten hydrolysate. These fractions were evaluated for their potential growth enhancement and increased production effects of immunoglobulin in hybridoma cells in serum-free media. One specific fraction of the second SEC step for the soy protein hydrolysate and one fraction of the SEC step for the wheat gluten hydrolysate resulted in similar enhancing characteristics. The soy protein hydrolysate resulted in a 150% higher maximum cell density, after a 6 days incubation period and the wheat gluten hydrolysate resulted in a 180% increase in comparison to the control conditions without hydrolysate. The productivity for both hydrolysates was similar and higher, 235% when compared to the control conditions. This suggests a significant contribution of these hydrolysate fractions to the cell growth and expression of proteins.

The enhancing growth effects of soy and wheat gluten hydrolysate were also compared to a rapeseed hydrolysate in CHO cells [Farges-Haddani et al., 2006]. After extraction, precipitation, hydrolysis and a second precipitation by acid of the rapeseed hydrolysate, the supernatant was filtered by a 3 and a 1kDa ultrafiltration membrane with a desalting step and tested with CHO cells grown in serum-free media. Each fraction led to a different growth behaviour. While the pellet of the acidic precipitation showed a complete inhibition of the cell growth, the retentate of the 1kDa ultrafiltration showed the highest cell growth enhancement with $6*10^5$cells/mL maximum cell density (1.5x higher than the control). The sample obtained

after hydrolysis, supernatant of acidic precipitation, and 1kDa ultrafiltration permeate showed cell densities similar to the control and therefore did not significantly stimulate the cell growth. Therefore it was concluded that the separation of these stimulating peptides is an important factor in the enhancement of cell growth. Compared to soy and wheat gluten hydrolysates, the rapeseed hydrolysate resulted in the highest stimulating effect for cell growth.

Another plant derived protein hydrolysate, rice protein hydrolysate, tested by Sung et al. (2004) in CHO cell cultures, did not yield any significant growth stimulating effects in serum-free media.

Although non-animal derived protein hydrolysates are preferred for safety issues, animal protein hydrolysate sources are also good replacements for serum in mammalian cell culture. Different milk protein hydrolysates such as lactalbumin or caseins or meat digests such as Primatone RL are good supplements in mammalian cell media.

Lactalbumin and NZCase (casein hydrolysate (Lebrun et al, 2004)) have been compared to yeastolate in the insect cell line Sf-9 by Mendonça et al. (2007). Compared to the control with no hydrolysate present, the crude lactalbumin hydrolysate resulted in a 33% higher maximum cell density. The growth rate also increased when compared to the control. There was no measurable effect for the cultures supplemented with crude NZCase. The addition of the 30kDa permeate fraction of NZCase had a higher enhancing effect than the retentate. Although a growth enhancing effect was found for NZCase and lactalbumin, yeastolate showed the highest impact for growth stimulation.

Primatone RL is a meat digest and a complex mixture containing amino acids, oligopeptides, iron salts, lipids, and other trace low molecular weight substances. Primatone RL is also inexpensive. Schläger (1996) tested the effect of Primatone RL on a number of cell lines (different mouse hybridomas, a mouse myeloma, and a human promyelocytic leukaemia HL-60 cell line) in serum-containing and serum-free media. The mouse myeloma cell line showed an increased growth rate compared to a negative control without Primatone RL added, especially with the simultaneous addition of insulin and transferin to the media. For a mouse

hybridoma cell line expressing a monoclonal antibody, the addition of Primatone RL resulted in higher cell density than with the addition of serum. Also, the addition of Primatone RL, for the different cell lines, prolonged the duration of the stationary phase. This is of extreme interest when the expression of a therapeutic protein is secreted in a non-growth pattern.

These studies indicate that the addition of animal-derived protein hydrolysates to mammalian cell cultures enhances growth and would be a cheap additive in comparison to serum. In addition to animal-derived proteins, plant-derived or yeast-derived protein sources also show promising results in the supplementation of serum. However, the supplementation of media by protein hydrolysates does have a few drawbacks:

a) Not a chemically defined compound
b) Possible batch to batch variation

To overcome the first problem, the fractionation, characterization and isolation of protein hydrolysates could be used. It has been shown that the addition of fractionationated peptides based on ultrafiltration and SEC had a positive impact on animal cell culture. As peptides are similar in size, ultrafiltration is not an ideal method as it separates molecules according to size. Nanofiltration on the other hand could be a useful membrane method for the separation of peptides as the separation is based on both size and charge.

2.3 Separation of Peptides

As mentioned previsouly, the separation of protein hydrolysates has become more popular as protein hydrolysates are also used in the food industry for the supplementation or to overcome protein allergies. Also the separation of the peptides contained in a protein hydrolysate is often required to obtain peptides with functional or bioactive properties (generally smaller than 1kDa) [Chen et al., 1996, Moure et al., 2006]. The two major methods for the separation of peptides in relatively large quantities are nanofiltration and size exclusion chromatography (SEC) and will be discussed in the following sections.

2.3.1 Nanofiltration

Nanofiltration is a pressure driven membrane separation process based on size and charge. Figure 2.1 presents the filtration process characteristics of nanofiltration in comparison to microfiltration, ultrafiltration and reverse osmosis. The membranes possess some charged groups on the surface. The filtration is generally perfomed at relatively high pressure, typically higher than 1 MPa. The MWCO of the membranes used for nanofiltration applications is sometimes in the lower range of ultrafiltration membranes which would then be considered a loose nanofiltration membrane [Schäfer et al., 2005]. The molecular weight cut off (MWCO) of the membranes based either on thin film composite or cellulose acetate is usually between 100 and 1000 Da. Membranes based on cellulose acetate have poor chemical and biological stability and are often more prone to fouling. To counter this problem, membranes based on thin film composite were developed. Polyamide is a common polymer for thin film composite material that GE Healthcare, for example, uses for their thin film membranes. It is composed of a multilayer structure supported on a polysulfone layer [Favre et al., 2008].

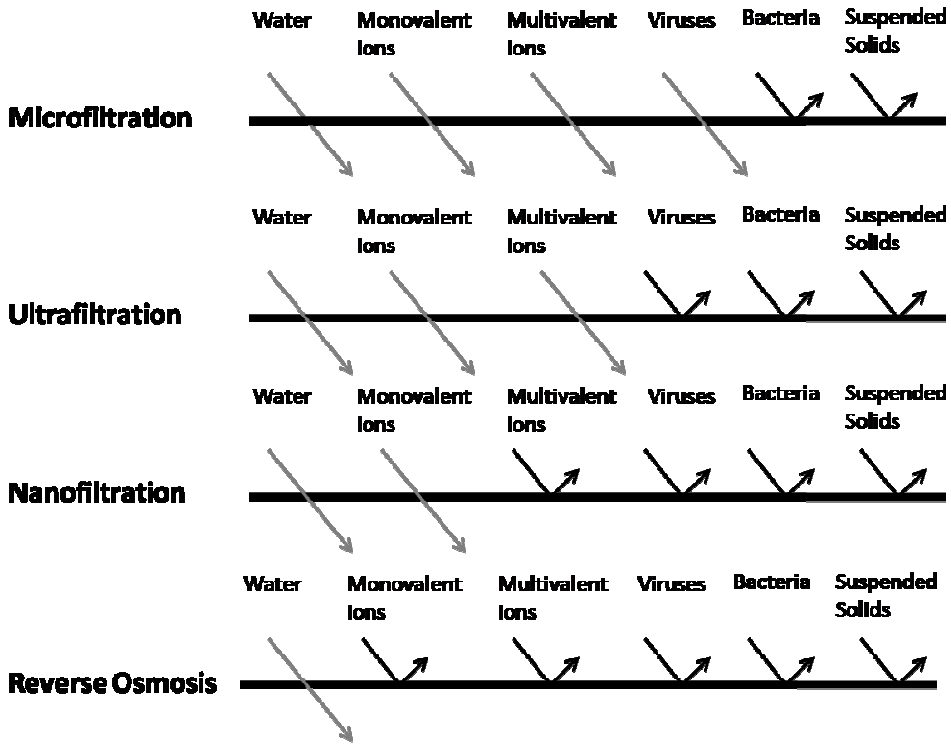

Figure 2.1 Filtration process characteristics of microfiltration, ultrafiltration, nanofiltration and reverse osmosis

A number of studies have shown the potential of nanofiltration for the separation of amino acids and peptides. The selectivity is based on the charge of amino acids, which influences the filtration process [Garem et al., 1997].

Initially developed for a desalting step of lactose in whey processing, nanofiltration has become more increasingly popular. Applications have a wide range including the paper, textile, food, dairy, water and chemical industry [Yacubowicz H. and Yacubowicz J., 2005].

Peptides have an amphoteric character which means that the molecule can either accept or donate a proton. It has been reported that amino acids and peptides similar in size but different in their net charge could be separated by a relatively loose nanofiltration membrane of around 1kDa [Schäfer et al., 2005]. The mechanism to separate molecules according to their charge is called the Donnan exclusion effect and is based on the charge interactions between the charged membrane and charged components [Pouliot et al., 1999]. This can lead to a separation between positively charged molecules and neutral or negatively charged molecules.

Several authors have investigated the separation of protein hydrolysates by nanofiltration. Tryptic hydrolysates of β-lactoglobulin and the effect of fouling by peptide-peptide interactions between hydrophobic peptides during nanofiltration were investigated by Groleau et al. (2004). The removal of peptide aggregation was performed by acid precipitation and the subsequent nanofiltration was carried out at pH 5 and 9. According to the results, the acidic treatment of the hydrolysate showed a significant increase of the permeate flux at pH 9, but no significant effect at pH 5. These results suggest that peptide aggregation did not affect filtration performance. It has been suggested that aggregates, or larger molecular weight components, have a positive effect in protecting the membrane from early fouling.

The influence of charged peptides in a whey protein hydrolysate was studied for different nanofiltration membranes at pH 5 and 9 with or without the addition of 500 mM NaCl [Pouliot et al., 1999]. The nitrogen transmission, the permeate flux, and the fouling resistance were the major parameters considered during the nanofiltration performed with 5 different membranes, based on cellulose acetate or thin film composite material. The SG13 membrane (cellulose

acetate, MWCO between 1 and 5kDa) showed the best filtration performance in terms of a moderate nitrogen transmission, 23% (at pH 5) and 35% (at pH 9), and a moderate permeate flux. The addition of 500 mM NaCl resulted in a higher nitrogen transmission at both pH 5 and 9. While the salt did not affect the permeate flux at pH 5, the permeate flux decreased significantly at pH 9. The highest selectivity of basic/acidic peptides was obtained for the permeate fraction at pH 9 without salt.

Tessier et al. (2006) investigated nanofiltration as a desalting step between the 3 and 1kDa ultrafiltration steps during the fractionation of rapeseed protein hydrolysates. The retentate fraction of the nanofiltration at pH 4 and 9 showed a reduced conductivity, around 80%, whereas the nitrogen content showed a 13% decrease. Furthermore, 40% of the nitrogen content for the permeate fraction was free amino acids. Therefore it can be assumed that the smaller molecules have a better transmission and the separation according to the size had a higher influence than the charge. On the other hand, capillary electrophoresis analysis of the nanofiltration fractions showed a higher content of basic peptides in the permeate fraction compared to the retentate fraction. For the acidic peptides, it was the opposite. According to these results, the charge of the membrane still showed a small effect on transmission. However, this specific membrane had a low peptide transmission in general, therefore the Donnan effect was assumed to have a limited impact in this case.

No publications are available on the fractionation of protein hydrolysates by nanofiltration for subsequent use as a supplement for serum in animal cell culture. Moreover no work has been done on the investigation of the fractionation of primatone and yeast extract by nanofiltration.

2.3.2 Size Exclusion Chromatography

As mentioned previously, size exclusion chromatography (SEC) can be used to separate peptides. This method separates molecules according to their molecular size. The packing material consists of beads with different pore sizes. This leads to an interaction between the molecules and the packing material of differing time duration and therefore results in different elution times. As peptides are often similar in size, the resolution of a SEC column is usually not high enough. Therefore a partial resolution is obtained. This situation is illustrated for the separation of wheat gluten hydrolysate by SEC investigated by Franek et al. (2000). The

separation was only partial; overlapping peaks were obtained because the peptides were similar in size. This being said, the different fractions of wheat gluten hydrolysates obtained by SEC and used as a supplement to serum-free media were still successful and yielded different growth behaviours in the cell cultures.

It can be concluded that a partial separation of peptides based predominantly on size is possible by SEC. If the objective of the separation is based on charge differences of the peptides, then nanofiltration would be the prefered method. As peptides with different charges may influence the cell culture, it would be wise to separate peptides according to their charge and investigate their influence on cell culture.

2.4 Analysis of Peptides

The development of a process for the separation of a mixture of peptides requires an analysis of the specific peptides obtained during the operation. In general the separation of peptides is more complicated than the separation of proteins because of the smaller size and the smaller differences between the different peptides. Therefore a sensitive method is required. The selection of the method will depend on the amount of sample available, the time constraints and the type of information desired. If the exact molecular weight of the different peptides contained in a mixture is of interest, then a method with mass spectrometry is necessary. Other less sensitive methods include: reverse phase (RP)-HPLC, which separates peptides according to their hydrophobicity; size exclusion chromatography (SEC)-HPLC, which separates peptides according to their size (but is limited because of the resolution of the specific column), capillary electrophoresis, which separates peptides based on their electrophoretic mobility; and OPA (o-phtaldialdehyde) assay which gives quantitative estimates of the peptide concentration, but does not distinguish individual peptides. This OPA method consists of the reaction between o-phtaldialdehyde and primary amines in presence of β-mercaptoethanol to a ring system which can easily be detected and measured at 340nm with a spectrophotometer (Figure 2.2). The peptide concentration is then given in equivalent phenylglycin, as the standard curve is performed with phenylglycin as a peptide.

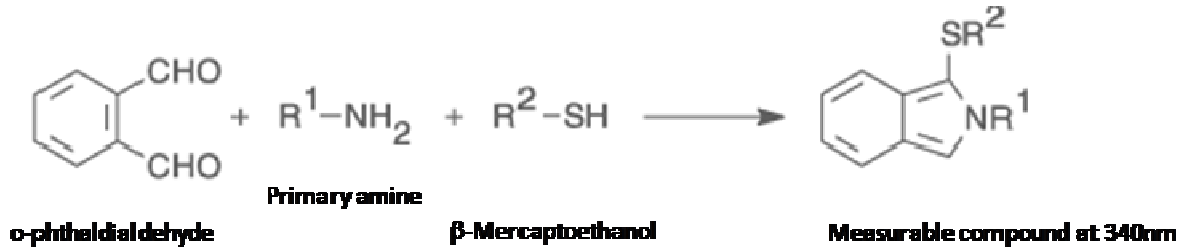

Figure 2.2 Reaction mechanism of the OPA assay in which primary amines react with OPA and b-mercaptoethanol to a ring system that is measurable at 340nm

RP-HPLC and mass spectrometry are the most common methods for the analysis of peptides, therefore these two methods will be described in more detail in the next sections.

2.4.1 RP-HPLC

Reversed phase-HPLC is the separation of molecules according to their hydrophobicity using a non-polar (hydrophobic) column and a moderately polar (hydrophilic) mobile phase. By increasing the gradient of the non-polar component of the eluent over time, the different molecules will differentiate themselves according to their respective ability to interact with the solid phase. Regarding the non-polar matrix of the column, the more hydrophobic character a molecule will possess, the longer its interaction with the stationary phase and the longer the retention time will be [Dong, 2006]. Various properties of the peptides will influence their interactions with the column including the amino acid composition, peptide chain length, and the sequence-dependent effects. These differences are the key to achieve the separation of the different peptides contained in a mixture [Tripet et al., 2007].

In analyzing biomolecules via HPLC, the detector is most often based on absorption in the ultraviolet or visible spectrum. To be able to obtain signals from the molecules, it is necessary that the molecules contain chromophores, which result from an energy difference between atomic orbitals. For the analysis of amino acids, peptides, or proteins there are only four amino acids, phenylalanine, histidine, tryptophan, and tyrosine, which contain chromophoric character. Therefore a derivatization of the peptides is often necessary to be able to detect them by UV-spectrometry. The purpose of the derivatization is to attach a chromopheric compound to the N-termini of each peptide or amino acid present in the sample.

Ferreira et al. (2005) used RP-HPLC for the quantitative determination of the two peptides Iso-Pro-Pro (IPP) and Val-Pro-Pro (VPP). Both peptides have angiotensin converting enzyme (ACE) inhibitory activity and can be found in β-casein hydrolysates. The separation of fermented milk samples was performed on a column based on divinylbenzene-copolymer and the gradient for the two eluents, 0.1% Trifluoroacetic acid (TFA) in water (A) and 0.1% TFA in acetonitrile (B), was carried out until a 50% concentration was reached in a 50 minute period. The ability to achieve reproducible and accurate peptide concentrations in a given sample was concluded to be very good. Therefore this method was appropriate for the quantitative estimation of the concentration of VPP and IPP by UV-detection and could be extended to other proteins and peptides.

The prediction of the retention time of peptides would improve the applicability of RP-HPLC to the identification of peptide mixtures of unknown composition. Tripet et al. (2007) investigated the influence of the hydrophobicity and hydrophilicity of the side-chains at the C- and N- termini of different peptides by changing the end-groups of all 20 amino acids that occur naturally to obtain a model that would predict retention times of peptides. To be able to determine the retention time of peptides, the whole sequence with its side-chains, interior amino acid composition, and the amino acid at the C- and N-termini are important and influence the analysis. Tripet et al. (2007) concluded that for the prediction of retention times of peptides, the knowledge of the composition of the respective peptide is important and is not possible with unknown peptides.

The analysis of peptides by RP-HPLC seems promising as a highly reproducible and accurate method; however this method has limitations when analyzing peptide mixtures of unknown composition. Also, if the mass and respective molecular weight of an unknown peptide are of importance, mass spectrometry is the only available method.

2.1.1 Mass Spectrometry

Mass spectrometry is the most sensitive method to identify molecules at the pico- and femtomol level. Even the difference of one amino acid in a sequence of a peptide can be analyzed. Nowadays, a fingerprint of peptides can be established through a database of all possible peptide sequences [Wanner and Höfner, 2007].

For the separation of proteins and peptides, chromatography for the separation and mass spectrometry for identification can be combined in many different configurations as described in the review by Careri and Mangia (2003). Liquid chromatography (LC) combined with a mass spectrometer is often the choice for the analysis of biomolecules such as proteins and peptides. To improve the analysis of peptides with similar size, a RP-HPLC with a mass spectrometry detector is used and provides quite accurate estimation of the mass of individual peptides. Because mass spectrometry is a lot more sensitive than a UV-detector it is possible to determine: the molecular weight of different compounds of low concentration; the peptide mass fingerprint; identification of the post-translational modifications of proteins; and the study of protein-protein or protein-ligand interactions. A matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) system in addition to a time-of-flight (TOF) detector have been used to determine the mass of proteins or peptides. In these methods a laser beam evaporates the solid compounds and generates ions that are injected in a tube, accelerated, and then allowed to drift toward a detector. The molecular weight is then calculated based on the time taken to reach the detector (time of flight) [Wanner and Höfner, 2007]. This method is often used in proteomics for the analysis of protein and peptide mixtures or even protein hydrolysate [Wanner and Höfner, 2007].

Shown by Bantscheff et al. (2007), mass spectrometry can also assist in the quantitative analysis of protein and peptide samples. It is still a challenge however, to obtain reliable results regarding quantification of specific components within a sample since different components can have the same signal. To obtain more reliable results, the respective samples are often marked with radioactive isotopes.

Tessier et al. (2004) used LC-MS to characterize and successfully identify the peptides present after the hydrolysis of rapeseed proteins. With the mass obtained by the MS-detector it was possible to identify the sequence of the respective peptides.

Chapter 3: Objectives

The aim of this thesis is the fractionation of peptide sources by nanofiltration in order to find a possible media supplement for serum-free cell culture of CHO cells. Specifically one non-animal protein hydrolysate (yeast extract) and one animal protein hydrolysate (Primatone) were selected as potential media supplements. Enrichment of these protein hydrolysates by membrane nanofiltration was evaluated. The project was a collaboration between the University of Waterloo and the University of Manitoba. Specific objectives were as follows:

1) Characterization of 5 nanofiltration membranes:
   - surface charge and hydrophobicity
   - water flux, membrane resistance and total solids transmission

2) Fractionation of yeast extract and Primatone by nanofiltration:
   - development of a pretreatment method
   - evaluation of the effect of salt and pH for two different membranes
   - peptide fraction characterization by RP-HPLC

3) Evaluation of nanofiltration fractions as a viable media additive for CHO cells: (performed at the University of Manitoba by Dr. Mike Butler and Vincent Jung)
   - cell growth
   - β-interferon productivity

The thesis is presented as 5 chapters with the last chapter being the overall conclusion and recommendation of the presented work.

Chapter 4: Characterization of 5 different nanofiltration membranes is presented in this chapter.

Chapter 5: The pretreatment of yeast extract and Primatone by ultrafiltration.

Chapter 6: Fractionation of yeast extract and Primatone by nanofiltration for two different membranes, and different pH and NaCl conditions.

Chapter 7: Development of a RP-HPLC method for the characterization of peptides in yeast extract and Primatone fractions.

Chapter 8: The influence of the supplementation of a serum-free media with yeast extract and Primatone nanofiltration fractions on CHO cells is presented in this chapter.

Chapter 9: This chapter presents the significant results of this study together with the recommendations for future work.

Chapter 4: Characterization of Nanofiltration Membranes

Overview

The characterization of membranes is an important aspect when selecting a membrane for a specific application as the filtration performance often relates to the membrane properties. In this work, five different nanofiltration membranes were characterized for their zeta potential, contact angle, water flux, membrane resistance, and the transmission of total solids. The HL and the G-10 membrane, both thin film composite membranes, were chosen for the subsequent nanofiltration experiments due to their different characteristics of hydrophobicity, surface charge, transmission of total solids, and water permeate flux.

4.1 Introduction

Nanofiltration is used in water treatment and the food and biotechnology industries. Specific applications include the concentration of gelatin, the desalination or concentration of whey and the fractionation of charged low molecular weight molecules. [Yacubowicz H. and Yacubowicz J., 2005]

The membrane with its specific characteristics is a critical component of the separation process. A number of commercial nanofiltration membranes are available. In order to choose a membrane for a specific application, the surface charge and the hydrophobicity of the membrane are important characteristics to be considered, because separation using these membranes is based on the size and charge of the molecules.

Nanofiltration membranes are mainly based on cellulose acetate or thin film composite materials [Yacubowicz H. and Yacubowicz J., 2005]. The polymer of the thin film composite material is a polyamide and is made by a multilayer structure supported on a polysulfone layer [Favre et al., 2008 / Sbai et al., 2003 / Al-Amoudi et al., 2007].

Manufacturers of nanofiltration membranes will rarely provide detailed information on the membrane properties except the salt retention, chemical resistance, and permeate flux. Any additional information will require experimentation. Characterization of membranes is performed with different methods to determine properties such as the pore radius, molecular weight cut-off (MWCO), membrane thickness, surface charge, hydrophobicity. The zeta potential and contact angle are two common methods for the estimation of the surface charge and the hydrophobicity of a membrane respectively. The contact angle depends on the surface properties of the membrane by measuring the shape of a liquid drop when in contact with the surface of the membrane material as represented in Figure 4.1 [Tadmore, 2004]. A contact angle less than 90° is characteristic of an hydrophilic material while a contact angle higher than 90° is associated with an hydrophobic material [Feng et al., 2002].

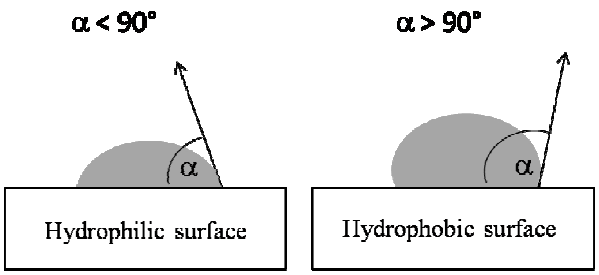

Figure 4.1 Shape of a water drop on either a hydrophobic or a hydrophilic surface with corresponding contact angle

The zeta potential provides information on the surface charge of a membrane. It describes the electric potential when a charged surface is placed in a solution containing dissolved ionic species. A schematic representation of the ionic species distribution around a negatively-charged membrane in a solution containing dissolved ionic species is presented in Figure 4.2. Two different layers are distinguished near the membrane surface, the immobile Stern layer, representing the ionic species with an opposite charge and fixed to the solid / liquid interface, and the mobile (diffuse) layer, in which the ionic species are moving and are both of negative and positive charge. The electric potential at the interface between the mobile layer and the immobile layer corresponding to a plane of shear is the zeta potential and is used to characterize the charge properties of a membrane surface [Burns and Zydney, 2000].

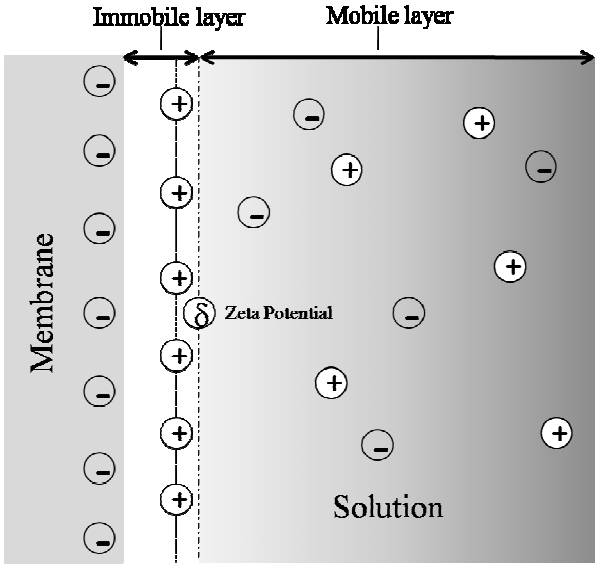

Figure 4.2 Schematic representation of the electrochemical double layer near a negatively-charged membrane surface

In this work, five different nanofiltration membranes, one cellulose acetate membrane (CK) and four thin film composite membranes (DL, HL, DK, G-10), were characterized according to their zeta potential, contact angle, total solids transmission for yeast extract as feed, membrane resistance, water flux and fouling potential.

4.2 Experimental

4.2.1 Chemicals

NaOH, KOH and HCl to adjust pH were of analytical grade. The water used for all experiments was obtained from a Millipore system (Synergy® Ultrapure Water system, Millipore, Etobicoke, ON, Canada) and had a conductivity of 0.056µS/cm.

4.2.2 Membranes

Four different nanofiltration membranes were purchased from Sterlitech (Kent, WA, USA) as flat sheets (30.5 x 30.5cm): DL / HL / DK / CK. The G-10 membrane was supplied by Filtration Engineering (Champlin, MN, USA). DL / HL / DK / G-10 are thin film composite material and CK is a cellulose acetate material. The specifications of the membranes provided by the manufacturer are summarized in Table 4.1. According to the literature, the DL, HL, and DK membranes have a MWCO between 150 and 300 Da [Amoudi et al., 2007]. The G-10 membrane is a loose nanofiltration membrane with a 2500 Da MWCO [Sbai et al., 2003]

Table 4.1 Membrane specifications provided by the manufacturer

Membrane	Material	$MgSO_4$ Rejection	pH range
CK	Cellulose Acetate	94%	2-8
DK	Thin film composite	98%	2-11
DL	Thin film composite	96%	2-11
HL	Thin film composite	98%	3-9
G-10	Thin film composite	n.a.	2-11

4.2.3 Contact Angle

The contact angle of each membrane was measured with a tantec contact angle meter (for more system information see US patent 5268 733, Glendale Heights, IL, USA), and a 5µL Millipore water drop placed on the membrane surface (n=15).

4.2.4 Zeta Potential

Zeta potential measurements were carried out with the SurPASS system (Anton Paar GmbH, Graz, Austria). Prior to each measurement, a new 5 x 2.5cm membrane sample was soaked in Millipore water overnight. The zeta potential of each membrane was then measured in 1mM KCl solution at a pH of 4 and 8 (to have the same conditions for the membranes as for the following nanofiltration experiments) by placing the membrane piece in the sample holder and measuring the streaming potential at different applied pressures (12 points in the range between 0 and 50kPa) (n=4).

The zeta potential (δ) was calculated with the following equation [Anton Paar, 2007]:

$$\delta = \frac{dU}{dP} * \frac{\eta}{\varepsilon * \varepsilon_0} * \frac{L}{A * R} \qquad (4.1)$$

Where $\frac{dU}{dP}$ = Slope of streaming potential versus pressure

$\eta = 8.90 \times 10^{-4}$ Pa*s (Electrolyte viscosity)

$\varepsilon_0 = 8.85*10^{-12}$ $C^2*N^{-1}*m^{-2}$ (Vacuum permittivity)

$\varepsilon = 78.54$ (dielectric constant of electrolyte)

L = length of the streaming channel [m]

A = cross-section of the streaming channel [m^2]

R = AC resistance across the measuring cell [Ω]

4.2.5 Experimental Nanofiltration Set-up

A schematic diagram of the filtration set-up is shown in Figure 4.3. The membrane holder was a SEPA CF II purchased from Sterlitech (Kent, USA) with an effective membrane area of 140 cm^2. The temperature of the feed was kept constant at 24°C by circulating water through a chiller (RTE-111, Neslab, Ottawa, Canada) to compensate for the heat generated by the diaphragm pump (M-03, Hydracell, Wanner engineering Inc., Minneapolis, MN, USA). A needle valve located on the retentate side was used to vary the transmembrane pressure (TMP).

The TMP was estimated from two pressure gauges (PGI-63B-MG1-LAQ1-A, Swagelok, Sarnia, ON, Canada), one located at the inlet and one located at the retentate side. The permeate was collected in a separate reservoir placed on a balance (Symmetry ML-10000-014, Cole Parmer, Anjou, QC, Canada) to record the permeate mass and estimate the corresponding permeate flux. The balance was connected to a computer with which the data was recorded through Labview version 7.1 (National Instruments, Vaudreuil-Dorion, QC, Canada)

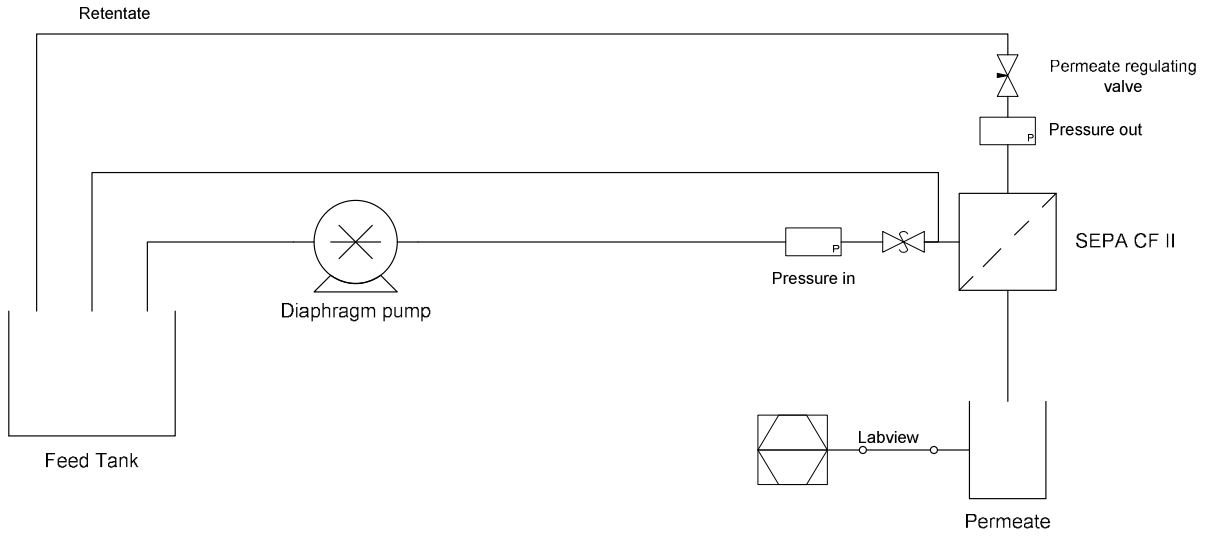

Figure 4.3 Schematic diagram of the nanofiltration set-up

4.2.6 Membrane Resistance and Water flux

The clean membrane resistance was obtained by measuring the water permeate flux of each membrane at 5 different pressures. The permeate water flux at a TMP of 2 MPa was used to represent the water flux for each membrane. The membrane resistance was determined as follows [Cheryan, 1998]:

$$R_m = \frac{TMP}{\eta * J} \tag{4.2}$$

Where R_m = Membrane resistance [m^{-1}]

TMP = Transmembrane Pressure [Pa]

η = viscosity of water [Pa*s]

J = Permeate flux [$m*s^{-1}$]

4.2.7 Transmission of Total Solids of Yeast Extract

Yeast Extract (Permeate of ultrafiltration experiment) was dissolved in 2 L Millipore water as a 0.1 %wt solution. Membranes were cleaned with 0.01 M NaOH for 30 minutes after each experiment to obtain the same water flux as before the filtration. When not being used, the membrane was stored at room temperature in Millipore water. All experiments were carried out at a constant TMP of 2 MPa, a flowrate of 1.8L/min, and at 24°C. The pH was adjusted to 8 by 1 M NaOH. Permeate was collected until half of the feed volume was filtered (VCR = 2). Both retentate and permeate fractions were stored at -20°C. The transmission of total solids was determined for the the feed, retentate and permeate and was calculated acccording to the following equation:

$$T_{TS}\,[\%] = \frac{TS_{Permeate}}{TS_{Retentate}}\,100\% \tag{4.3}$$

Where T_{TS} = Transmission of total solids

$TS_{Permeate}$ = Total solids contained in the permeate at the end of the filtration

$TS_{Retentate}$ = Total solids contained in the retentate at the end of the filtration

4.2.8 Fouling potential

The fouling of different membranes can be compared with an equation based on the initial and steady state permeate flux. A fouling potential according to equation 4.5 was used to compare the fouling characteristics of each membrane.

$$FP = \frac{J_0 - J_F}{J_0} \tag{4.4}$$

Where FP = Fouling potential [-]

J_0 = Initial permeate flux[LMH]

J_F = End (steady state) permeate [LMH]

4.2.9 Statistical Analysis

For the determination of a possible significant difference between two means, the t-test with one tail was used with a confidence interval of 95% (α = 0.05). The equation used to distinguish two means is as follows: [Walpole et al., 1998]

$$t = \frac{X_1 - X_2}{\sqrt{\frac{s_1^2}{n_1} + \frac{s_2^2}{n_2}}} \quad (4.6)$$

Where x = sample mean

s = standard deviation

n = number of samples

4.3 Results and Discussion

4.3.8 Contact Angle

The hydrophobicity of a membrane was measured by the contact angle of a water droplet. A hydrophobic membrane would be characterized by a contact angle between 90 and 180°. Membranes with contact angles smaller than 90° are considered hydrophilic [Feng et al., 2002]. According to this classification all of the nanofiltration membranes have hydrophilic properties. Furthermore, the lower the angle the higher is the hydrophilicity of the membrane material. With a contact angle of 34.3° the DK membrane is the most hydrophilic membrane. The cellulose acetate membrane with a 68.9° contact angle possesses the least hydrophilic characteristic. As visualized in Figure 4.4, the HL and DL membranes show statistically similar hydrophilicity (t-test: α = 0.05, one tail). All other membranes were statistically different from each other (t-test: α =;0.05, one tail). According to these results, the thin film composite membranes investigated in this study exhibit higher hydrophilicity than the cellulose acetate membrane. Furthermore, two of the tested thin film composite membranes show similar contact angles. This leads to the assumption that even if the contact angle is similar, the membrane properties can be still different. Therefore the contact angle is not the only important characteristic of a membrane.

Experimental measurements of the contact angles for the HL, DK, and DL membranes reported by other authors [Al-Amoudi et al., 2008] were found in the literature. Compared to these estimates, the contact angles of the HL and DL membranes obtained in this study are similar. In contrast, the contact angle of the DK membrane was 10° different. This difference can be due to the type of experimental set-up used in the two studies.

It has been shown that hydrophilic membranes adsorb less proteins on the surface which leads to a lower fouling of the membrane [Koehler et al., 2000]. Thus, the more hydrophilic the membrane the less fouling would be expected. The hydrophilic properties of the five membranes tested in this study are expected to possess low fouling characteristics and should be advantageous for a filtration process with peptides.

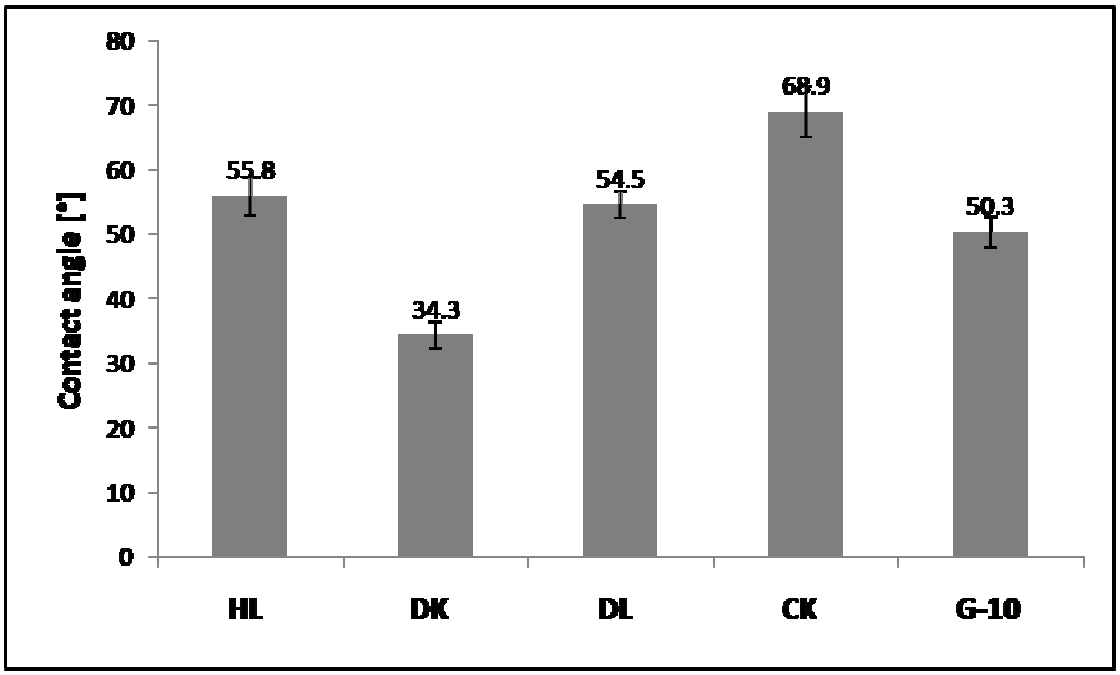

Figure 4.4 Contact angle of 5 different nanofiltration membranes (n=15)

4.3.2 Zeta Potential

Since the zeta potential gives an indication of the charge of the membrane surface it depends on the ions in the solution and on the properties of the membrane surface. As it depends on the ions in the solution, the zeta potential will change according to the pH conditions. To illustrate this characteristic, each membrane was investigated at two different pH, pH 4 and pH 8. These two pH‘s present an acidic and a basic condition and were therefore chosen for the following nanofiltration experiments.

According to Figure 4.5, the zeta potential for each membrane was affected by the pH. The G-10 membrane shows the smallest difference, but this difference is still statistically different (t-

test: $\alpha = 0.05$, one tail). At pH 4, all membranes show a higher zeta potential than at pH 8. The G-10 membrane is the only membrane with a negative zeta potential at both pH. A negative charge at pH 8 and a positive charge at pH 4 was obtained for the CK, HL and DL membranes. The DK membrane had a positive zeta potential at both pH and the highest positive zeta potential of all the membranes tested. Good experimental reproducibility was obtained with the standard deviation ranging between 1 and 6% for four replicates.

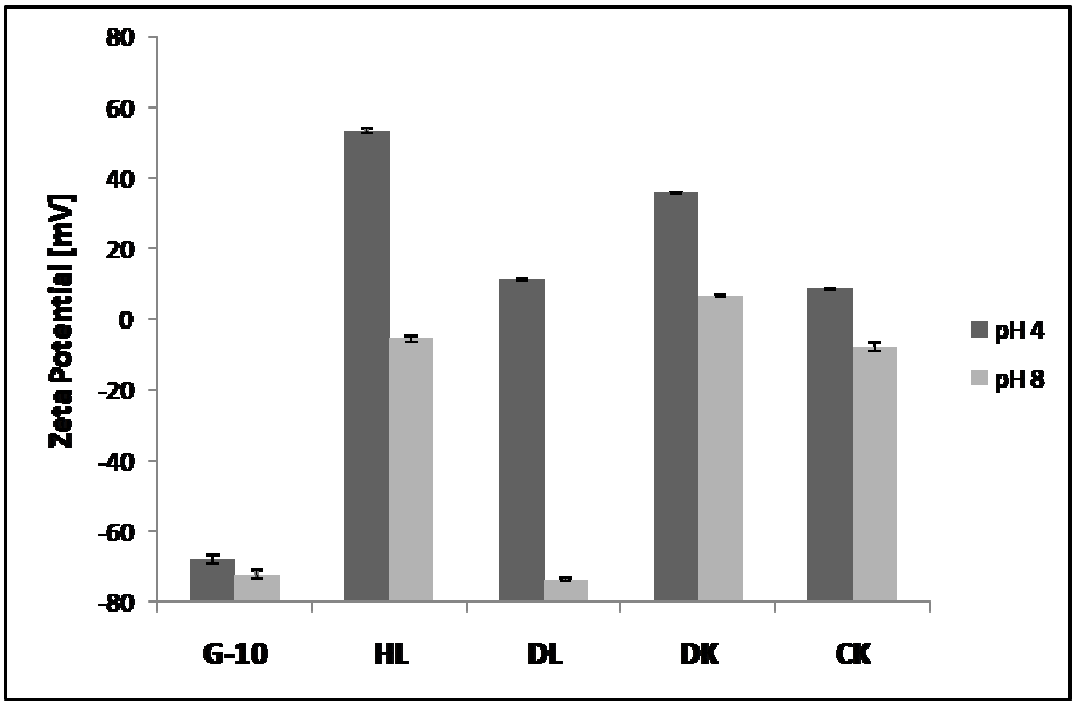

Figure 4.5 Zeta Potential of 5 different nanofiltration membranes (n=4)

According to literature, nanofiltration membranes have mostly negatively charged characteristics at neutral pH [Yacubowicz H. and Yacubowicz J., 2005]. The zeta potential was not determined at a neutral pH and according to some publications the linearity or non-linearity depends on the membrane [Nyström et al., 1994, Burns and Zydney, 2000]. Therefore it can not be concluded if the tested membranes have a negative charge at a neutral pH. But all membranes except the DK membrane showed a negative potential at pH 8 and therefore it is assumed to be still negative at a pH of 7.

All five membranes investigated in this study have different zeta potential and correspondingly surface charge. It was shown by Nyström et al. (1994) that the zeta potential is related to the pore size of a membrane, where smaller pores result in a lower zeta potential. This was also observed in this study when comparing the G-10 membrane to the other four membranes. The G-10 membrane with the highest reported MWCO displayed the most negative zeta potential One would expect that these differences would be reflected during the nanofiltration operations.

4.3.3 Membrane Resistance and Water Permeate Flux

The membrane resistance corresponds to the resistance of the water permeation through the membrane and should be independent of the pressure. Nanofiltration membranes are tight membranes, therefore high resistance to permeation and correspondingly a low water permeate flux is expected.

According to Table 4.2, each membrane has distinct membrane resistance and water permeate flux. The HL membrane shows the lowest membrane resistance and therefore the highest water permeate flux. Although the G-10 membrane is a loose nanofiltration membrane with a significantly higher MWCO than the HL membrane, it did not result in the highest water permeate flux. Instead the G-10 membrane had the lowest water permeate flux of the four thin film composite membranes investigated in this study. The cellulose acetate membrane (CK) showed the highest resistance and therefore also the lowest water permeate flux. It can be concluded that cellulose acetate as a membrane material yields a higher membrane resistance and thus a lower water permeate flux than the thin film composite membranes. The differences in membrane resistance and water permeate flux demonstrate different membrane properties and that the membrane MWCO is not always related to the water permeate flux.

Al-Amoudi et al. (2008) proposed a relationship between the contact angle and the water flux for three of the membranes investigated in this study (HL, DK, and DL). But no relationship between the contact angle and the water flux was found in the current study. Plots of water flux or membrane resistance versus contact angle and zeta potential can be found in the Appendix 2. The contact angle were similar, but as the membranes are probably not from the

same batch it is possible that the properties are slightly different and have influenced the water permeate flux. Furthermore different anlaysis techniques were used in the two studies.

Table 4.2 Membrane resistance (R_m) and water permeate flux (J_w) at a TMP of 2 MPa of different nanofiltration membranes

Membrane	R_m [10^{-12} m^{-1}]	J_w [$m*s^{-1}$]
DK	4.0	5.0
DL	4.7	4.3
HL	1.8	10.94
CK	9.6	2.1
G-10	6.9	2.9

4.3.4 Transmission of total solids

The transmission of a molecule is the ability of this molecule to pass through the membrane. It is an important parameter for the characterization of the membrane performance for both concentration or fractionation operations.

The G-10 membrane showed the highest transmission of total solids with 23%. All other membranes displayed a very low transmission of total solids ranging between 5 and 8%. The significant difference of the transmission of total solids between the G-10 membrane and the other membranes suggest different properties of the membrane and could be related to its significantly higher MWCO of the G-10 and significantly lower zeta potential. Figure 4.6 presents the relationship between the transmission of total solids and the zeta potential at pH 8 and suggest that the G-10 membrane has a very different zeta potential at pH 8 in comparison to all other tested membranes. In the case of yeast extract fractionation, the separation process seems to be influenced more by the membrane pore size than the charge of the solids. But with a different pH or the addition of salt this behaviour may change for nanofiltration operation.

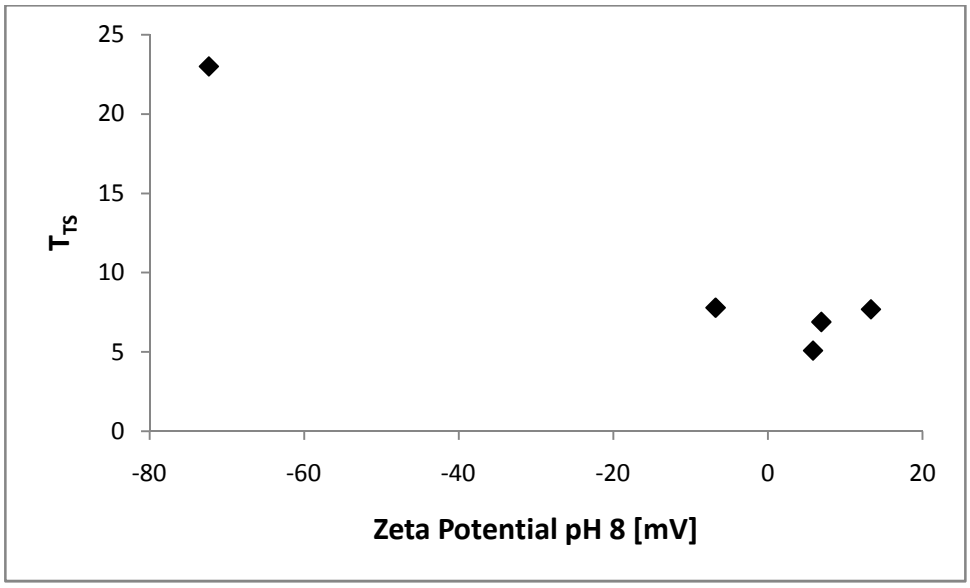

Figure 4.6 Relationship between zeta potential at pH 8 versus the transmission of total solids

4.3.5 Fouling Potential

The fouling potential of each membrane is presented in Table 4.3 and was calculated considering the initial and final (steady state) permeate flux. A fouling potential is of interest to compare the different fouling behaviours of different membranes. The membrane based on cellulose acetate showed the lowest fouling potential and the G-10 with the highest MWCO showed the highest fouling potential. According to these results the membrane with the highest fouling potential and one with a moderate fouling potential were chosen for the nanofiltration experiments.

Table 4.3 Fouling potential for four thin film composite membranes and one cellulose acetate membrane

Membrane	Fouling potential
DK	0.05
DL	0.14
HL	0.17
CA	0.01
G-10	0.27

4.4 Conclusions

In order to select two nanofiltration membranes for the fractionation of yeast extract, five different membranes, four thin film composite and one cellulose acetate membranes were characterized according to surface charge, hydrophobicity, water permeate flux, membrane resistance, transmission of total solids and fouling potential.

Although four membranes were thin film composite membranes and possesed hydrophilic properties deduced from the contact angle properties, each membrane still showed different zeta potential properties. The hydrophilic properties of the membranes are positive and should lead to low fouling in protein separations.

The two membranes selected for the subsequent nanofiltration experiments were the HL and G-10 membrane. Both membranes are thin film composite materials with very different MWCO. The MWCO reported in the literature of the HL membrane is between 150-300Da and around 2500Da for the G-10, a loose nanofiltration membrane. Both membranes show statistically significant different contact angle and zeta potential (t-test: $\alpha = 0.05$, one tail). At a pH of 4 the G-10 membrane has a strong negative surface charge while the HL membrane has a positive surface charge. The transmission of the total solids is the most distinctive property with a transmission of 23% of total solids in the permeate for the G-10 membrane compared to 8% for the HL membrane. This is most likely due to the higher MWCO of the G-10 membrane. Although the G-10 membrane has a higher MWCO than the HL membrane, the water permeate flux is lower and therefore the membrane resistance of the G-10 membrane is higher as well. The fouling potential also resulted differences between the two membranes. When comparing all these properties, one would expect different peptide fractionation behaviour for yeast extract when manipulating the pH and the ionic strength.

Chapter 5: Feed Preparation by Ultrafiltration

Overview

This chapter presents the ultrafiltration of yeast extract and Primatone for the removal of macromolecules prior to the nanofiltration experiments. The optimal conditions for the ultrafiltration operation were identified to be a 65 kPa TMP and a 5 wt% feed concentration. The higher fouling observed for yeast extract than for Primatone suggests a higher concentration of higher molecular weight components present in yeast extract. The fractions of the ultrafiltration were analyzed for peptide concentration by the OPA assay, the total solids present, and antioxidant capacity by the FCR assay. The permeate fraction contained about 70% of the peptides of both feed sources and was used for the subsequent nanofiltration experiments at different conditions.

5.1 Introduction

Nanofiltration is a membrane based separation process used for components smaller than 1kDa. A pretreatment step for nanofiltration is often necessary to remove high molecular weight molecules in order to protect the membrane, reduce fouling and improve membrane performance [Schäfer et al., 2005]. Several methods can be used prior to nanofiltration operations including microfiltration, ultrafiltration, chemical or coagulant ($FeCl_3$ for example) addition [Capar et al., 2007, Schäfer et al., 2001, Schäfer et al., 2005]. Coagulants such as $FeCl_3$ will precipitate potential foulants such as hydrophobic organic matter and calcium ions present in water [Schäfer et al., 2001].

Pretreatment in the water industry includes the addition of chlorine, ozone or UV-irradiation to prevent the growth of microorganisms [Schäfer et al., 2005]. Ultrafiltration is the common pretreatment for the nanofiltration of peptide mixtures and eliminates higher molecular weight molecules [Tessier et al., 2006, Pouliot et al.,1999, Groleau et al., 2004].

Ultrafiltration, the most important pretreatment method for protein nanofiltration applications, is also a pressure driven membrane separation process where a membrane with well defined porosity enables the separation of molecules ranging in size between 1 and 1000 kDa dissolved in a liquid stream [Cheryan, 1998] operating at moderate transmembrane pressures (dependent on membrane and application).

Primatone and yeast extract contain a mixture of proteins, peptides, lipids, vitamins, and minerals. In this study ultrafiltration with a 10k Da hollow fibre membrane was carried out as a pretreatment step, with the permeate to be collected and used for subsequent nanofiltration operations. The investigation of the different operating conditions for the ultrafiltration pretreatment step will be presented in this chapter.

5.2 Experimental

5.2.1 Chemicals

Folin & Ciocalteu's phenol reagent (FCR), o-phthaldialdehyde (OPA), β-mercaptoethanol, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox), sodium dodecyl sulfate (SDS), sodium carbonate were purchased from Sigma (Oakville, ON Canada). Yeast Extract (211929 lot# 8219390) was purchased from BD Bioscience (Mississauga, ON, Canada) and phenylglycin and Primatone RL (2751016 lot# R24429) from MP Biomedicals (Montréal, QC, Canada). The water used for all experiments was obtained from a Millipore system (Synergy® Ultrapure Water system, Millipore, Etobicoke, ON, Canada) with a conductivity of 0.056μS/cm.

5.2.2 Experimental Set-up

Ultrafiltration experiments were carried out using a 10 kDa polysulfone hollow fibre membrane with a membrane area of 420 cm^2 purchased from GE Healthcare (UFP-10-E-4MA, Baie d'Urfe, Canada). The filtrations were conducted at constant TMP, ambient temperature (24°C) and 2.4 L/min as feed flowrate. A schematic diagram of the filtration set-up is shown in Figure 5.1. The tangential flow filtration set-up consists of a flowmeter (Cole Parmer, Anjou, QC, Canada) to measure the flowrate and pressure transducers (Cole Parmer, Anjou, QC, Canada) on the feed and retentate side to determine the transmembrane pressure (TMP). The pinch valve (Cole Parmer, Anjou, QC, Canada) located on the retentate line was used to adjust the TMP. A progressing cavity pump (Moyno Inc., Springfield, OH, USA) was used to pump the feed into the set-up. The permeate was collected in a separate reservoir placed on a balance to record the permeate mass and estimate the corresponding permeate flux. The balance and pressure transducer were connected to a computer and the data recorded using Labview version 7.1 (National Instruments, Vaudreuil-Dorion, QC, Canada).

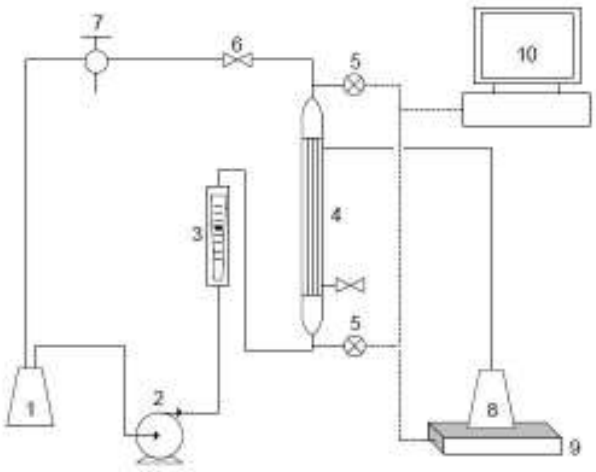

Figure 5.1 Schematic diagram of the ultrafiltration set-up: 1 feed tank, 2 pump, 3 flowmeter, 4 membrane, 5 pressure transducers, 6 pinch valve, 7 sampling valve, 8 permeate container, 9 balance, and 10 PC/software

5.2.3 Identification of Operating Parameters

To find an appropriate processing TMP and feed concentration, the permeate flux was calculated from the mass of the permeate collected at different time intervals for different pressures. Two feed concentrations were investigated for yeast extract and Primatone, 1 and 5% wt.

5.2.4 Ultrafiltration Experiments

Yeast extract and Primatone were dissolved in Millipore water as a 5 %wt feed solution. The ultrafiltration experiments were carried out at a constant transmembrane pressure (TMP) of 65 kPa, a flowrate of 2.4 L/min, and at room temperature (24°C). The permeate was collected until 2/3 of the feed was filtered (VCR=3).

The volume concentration ratio (VCR) is calculated according to the following equation [Cheryan, 1998]:

$$VCR = \frac{V_F}{V_F - V_P} \quad (5.1)$$

Where VCR = Volume concentration ratio

V_F = Volume of feed

V_P = Volume of permeate

Aliquoted 50 mL samples of the permeate were stored at -20°C. The total solids, the antioxidant capacity by FCR assay, and the peptide concentration by OPA assay were determined for the feed, retentate and permeate.

5.2.5 Total Solids

The total solids content of the feed, retentate, and permeate fractions were analyzed by estimating the dry weight of a 10mL sample (n=2) placed overnight in an oven at 100°C (Hotpack, Waterloo, ON, Canada).

5.2.6 Total peptide concentration assay (OPA)

The OPA solution was prepared by combining 50 mL of 80 mM sodium borate decahydrate, 20 mL of 10 % SDS, 80 mg of OPA in 2 mL ethanol, and 200 μL β-mercaptoethanol to a total volume of 100 mL. The pH was adjusted to 9 with 1 M HCl.

The standard curve was prepared using phenylglycin at concentrations of 0 / 250 / 500 / 750 / 1000 μM.

A volume of 10 μL of a 1:20 dilution (5 μL sample in 95 μL Millipore water) of the feed, retentate, and permeate sample and 100 μL of the standards were added to 1 mL OPA solution, swirled by inversion, incubated for 2 min at room temperature and the absorbance measured at 340 nm with a spectrophotometer (Cary 1Bio, Mississauga, ON, Canada). Each sample was prepared in duplicate.

5.2.7 Antioxidant capacity assay (FCR)

For the analysis of the feed, retentate, and permeate, the samples were diluted 1:4 (10 μL sample and 30 μL Millipore water) and were carried out in duplicates.

As a standard, trolox dissolved in ethanol was used. The linearity for the standard curve was between 0 and 3 mM. Five different Trolox concentrations were prepared to obtain a standard curve (1 / 1.5 / 2 / 2.5 / 3 mM).

A volume of 20 μL of each sample or standard solution was added to a 4 mL cuvette. After the addition of 150 μL of FCR solution, the cuvettes were incubated for 5 minutes at room temperature. After the incubation time, 600 μL of 15 %wt sodium carbonate (Na_2CO_3) and 2230 μL of Millipore water were added and swirled by inversion After a 2h incubation time,

the absorbance was read at 750nm with a spectrophotometer (Cary 1Bio, ON, Mississauga, Canada).

5.2.8 Freeze drying

The freeze drying was performed with 5mL of each feed, retentate, and permeate fraction in a Labconco Freezone 6 (Kansas City, MI, USA) system. The samples were first frozen at -20°C and then freeze dried for 3 days at -50°C.

5.3 Results and Discussion

5.3.1 Identification of Operation Parameters

For both feed sources, the two different concentrations investigated in this study (Figure 5.2 and Figure 5.3), a linear relationship was observed between the permeate flux and the transmembrane pressure when the permeate was recycled back to the feed tank (total recycle operation). This behaviour indicates that negligible fouling occurs. As presented in Figure 5.2 for yeast extract, increasing the feed concentration had a negative influence on the permeate flux and resulted in a lower permeate flux. In contrast for Primatone (Figure 5.3), increasing the feed concentration had no effect on the permeate flux. In fact Primatone and pure water showed similar TMP/flux relationships. According to these results the parameters for the pretreatment of yeast extract and Primatone by UF were chosen. Since increasing the feed concentration did not dramatically decrease the permeate flux in either case, the feed concentration was chosen at 5 %wt for both feed sources. The TMP was set at 65 kPa which represents realistic operation conditions well below the 110 kPa pressure limit of the membrane unit.

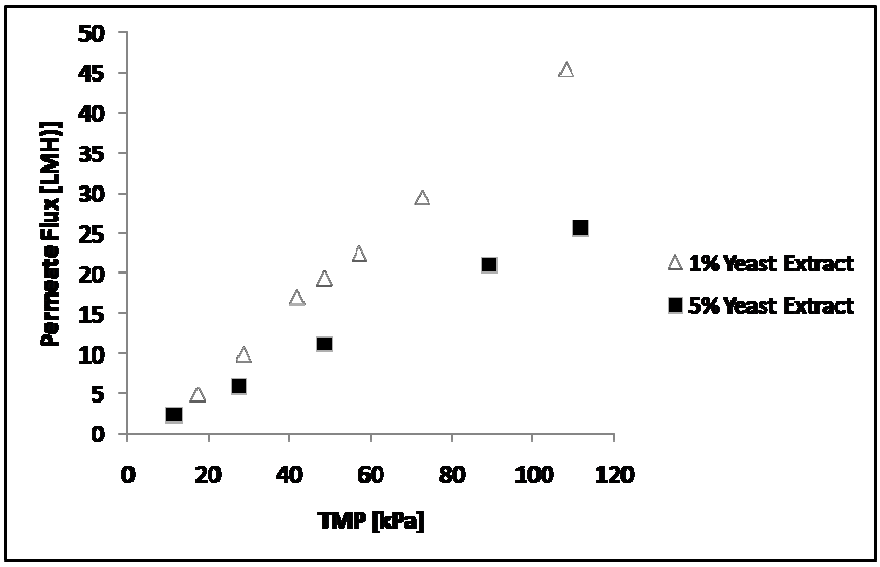

Figure 5.2 Permeate flux vs transmembrane pressure profile (total recycle operation) of yeast extract at two different feed concentrations

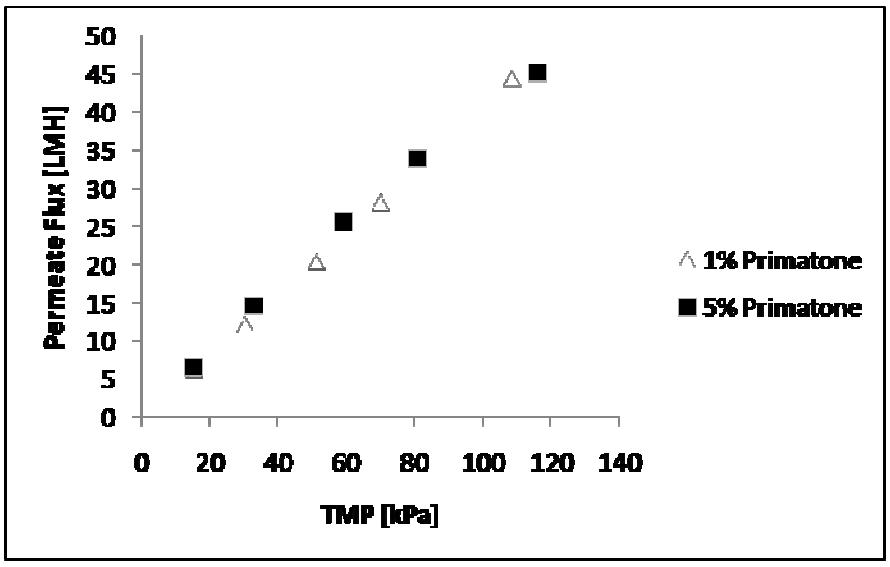

Figure 5.3 Permeate flux vs transmembrane pressure profile (total recycle operation) of Primatone at two different feed concentrations

5.3.2 Filtration Performance of Yeast Extract and Primatone

Figure 5.4 presents the filtration performance of yeast extract and Primatone over time for concentration operation (ie the permeate is continuously removed as the filtration proceeds). The permeate flux for yeast extract was always lower (20 L/(m^2*h) (LMH) initially leveling to ~ 12 LMH at the end of the operation) than for Primatone (relatively constant at ~ 25 LMH). This indicates higher fouling from yeast extract caused by the components retained at the

membrane surface (ie larger than 10 kDa). These differences agree with the total solids distribution between the retentate and permeate, with 34% total solids in the retentate for yeast extract and 28% for primatone (Table 5.1 and 5.2) indicating a lower amount of components smaller than 10 kDa for Primatone. Both feed sources are heterogeneous digested materials from two different sources yeast cells and animal tissues; therefore, it can be assumed and expected that the content of the two sources would differ.

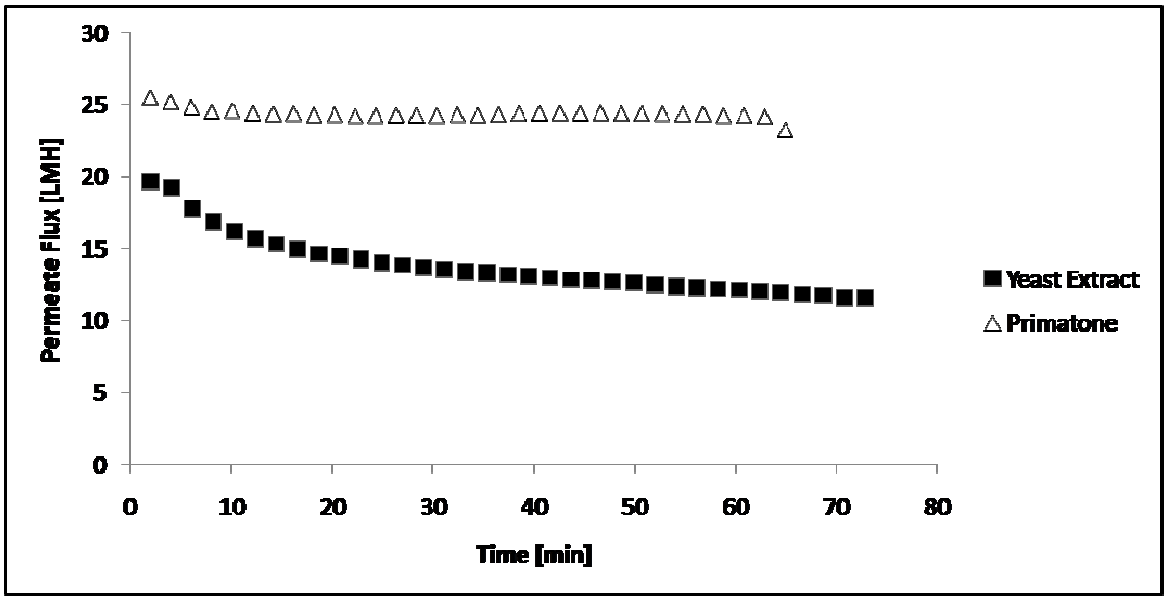

Figure 5.4 Filtration concentration operation at TMP= 65 kPa of 5 %wt yeast extract and 5 %wt Primatone

The peptide concentration (equivalent phenylglycine concentration), and antioxidant capacity (mg Trolox/mg of sample) for the feed, retentate, and permeate are presented in Table 5.1 and Table 5.2. Both feed sources had similar total peptide content, about 635 mg peptides/g of total solids and similar permeation of total peptides (about 70% in the permate). This being said, the OPA assay does not distinguish individual peptides. The mass balance for the total solids and peptide concentration for both feed types are in the expected range of ± 5%. The antioxidant capacity could not be concentrated in the permeate for both yeast extract and Primatone. This is most likely due to a large standard deviation between the replicates, suggesting the method is not reproducible enough for low concentrations of antioxidants present in the sample. Also, according to the literature small peptides (containing between 2 and 16 amino acid residues) are considered to show more antioxidant activity [Chen et al., 1996, Moure et al., 2006] and may not have been fractionated when using 10 kDa ultrafiltration membranes.

Table 5.1 Peptide concentration, total solids, and antioxidant activity of the feed, retentate, and permeate sample of the yeast extract filtration

Fraction	Equiv PheGly* [mg/g solids]	Equiv PheGly* mass [%]	Total solids [g/L]	Total solids [%]	Antioxidant activity [mg Trolox/mg sample]
Feed	639.4 ± 26.1	100.0	43.7 ± 2.3	100.0	71.0 ± 11.1
Retentate	632.7 ± 12.5	33.6	50.1 ± 1.6	34.0	63.7 ± 19.1
Permeate	732.2 ± 35.2	70.6	38.3 ± 0.6	61.6	79.6 ± 5.9
Total**		104.2		95.6	
Loss		4.2		-4.4	

* Equiv PheGly = Equivalent Phenylglycin as relation to the peptide concentration

** Total = Sum of retentate + permeate

Table 5.2 Peptide concentration, total solids, and antioxidant activity of the feed, retentate, and permeate sample of the primatone filtration

Fraction	Equiv PheGly* [mg/g solids]	Equiv PheGly* mass [%]	Total solids [g/L]	Total solids [%]	Antioxidant activity [mg Trolox/mg sample]
Feed	630.4 ± 36.2	100.0	47.3 ± 2.1	100.0	96.4 ± 16.2
Retentate	584.4 ± 8.5	26.1	52.6 ± 2.4	28.2	88.4 ± 17.3
Permeate	672.2 ± 14.1	72.8	43.2 ± 2.0	68.3	108.0 ± 20.5
Total**		98.9		96.5	
Loss		-1.1		-3.5	

* Equiv PheGly = Equivalent Phenylglycin as relation to the peptide concentration

** Total = Sum of retentate + permeate

5.4 Conclusions

The appropriate operating conditions for the 10 kDa UF membrane pretreatment step were identified to be a 65 kPa TMP and a 5 wt% feed concentration according to the permeate flux vs transmembrane pressure profiles. Using these operating conditions, yeast extract and Primatone were filtered with the permeate continuoulsy removed until 33% of the feed volume had been reduced. This operation resulted in the recovery of around 70% of the initial equivalent peptides in the permeate for both feed sources. A significantly difference filtration behaviour was observed between Primatone and yeast extract. Yeast extract showed significantly more fouling than Primatone, which may be associated with the higher total solids content in the retentate for yeast extract and the difference in composition of the two feed sources suggesting a higher content of high molecular weight components for yeast extract. The antioxidant capacity does not appear to be significantly concentrated in the permeate for both feed sources. But a large standard deviation between the replicates was also observed. The collected UF permeate for Primatone and yeast extract will be used in the subsequent nanofiltration study.

Chapter 6: Nanofiltration of Peptides

Overview

Nanofiltration of peptide mixture is a newer application and is still not fully understood. The investigation of different parameters is therefore an opportunity to obtain a better knowledge of peptide fractionation by nanofiltration. In the present work, the effect of pH (4 and 8), and the presence or absence of salt on the filtration performance was investigated with a 2^4 factorial design for two different membranes (HL and G-10) and two feed sources (yeast extract and Primatone). The significant factors were obtained by creating linear regression models with factors that showed a p-value <0.05. The two membranes showed very different filtration performance. The G-10 membrane had a 30% total peptide transmission and the HL membrane showed only a 10% total peptide transmission even though the permeate flux was higher for the HL membrane which has a smaller reported MWCO than the G-10 membrane. Furthermore different effects of NaCl and pH for the two feed sources and the two membrane types were observed. This suggests that nanofiltration is dependent on the charge and ionic strength of the solution and constitutes a promising method for the fractionation of peptides contained in yeast extract and Primatone.

6.1 Introduction

Filtration is a commonly used technique for the physical separation of molecules with different sizes. Most filtration processes, such as microfiltration and ultrafiltration are based exclusively on size difference. However, nanofiltration separates molecules according to their size and their charge [Schäfer et al., 2005]. The ability to separate molecules according to size and charge is an important factor for the separation of peptide mixtures. Peptides are often similar in size and can not be separated completely by ultrafiltration or SEC. Although the purification and separation of peptides by chromatography according to the charge or hydrophobicity is a possible method, it is not feasible for large scale at a reasonable cost. Chromatography in industrial processes is time-intensive, costly, and can be difficult to scale-up. In contrast, filtration processes have relatively simple operating procedures, are less expensive and relatively easy to scale-up [Chmiel, 2006]. Depending on the membrane it is also less time-intensive.

Nanofiltration has only been used in industrial applications since the late 1970's. Nowadays, applications of nanofiltration processes are found in the dairy-, food-, and chemical industry as well as in the water treatment [Yacubowicz H. and Yacubowicz J., 2005]. The fractionation of peptide mixtures by nanofiltration has been investigated by a number of authors in the last decade. Pouliot et al. (1999) illustrated the importance of pH and NaCl concentration during the fractionation of tryptic hydrolysates of whey proteins by nanofiltration. Groleau et al. (2004) showed the feasibility to fractionate peptides contained in the tryptic hydrolysates of β-lactoglobulin according to their size and charge by manipulating the pH. No publications are available for the fractionation by nanofiltration of the peptides contained in yeast extract or Primatone.

A number of authors have shown the beneficial effect of the addition of a protein hydrolysate of animal or non-animal source on the growth of mammalian cells as a substitute to serum. Promising sources of protein hydrolysates include rapeseed protein hydrolysate, soy protein hydrolysate, wheat gluten, Primatone, or yeastolate [Schläger 1996, Farges-Haddani et al. 2006, Franek et al. 2000, Sung et al. 2004]. As it is not known yet which peptides are growth

stimulating for mammalian cells, the next step is the characterization of the specific peptides responsible for the cell growth enhancement.

Nanofiltration represent an attractive approach for the concentration of a specific peptide or peptide group (for example negatively charged peptides) in either the permeate or retentate. A subsequent step for characterization of the permeate and retentate fraction would be necessary to identify the specific molecular weights and subsequent sequence of each peptide (for example by HPLC-MS).

Filtration performance is dependent on the applied pressure, the driving force for the separation; the temperature, which increases or decreases the viscosity and therefore affects the flux; the crossflow velocity, which indirectly affects the fouling; the pH, which is responsible for the charge of the membrane and the solution; and lastly, the ionic strength for the solution, which affects the interactions near the membrane.

In this study, two feed sources were investigated; yeast extract and Primatone. Yeast extract, obtained by autolysis or hydrolysis of yeast cells, is used in health food or as a nutritional supplement in microbiological media. Yeast extract is a non-animal product and contains a mixture of proteins, peptides, lipids, nucleotides, vitamins, and minerals [Chae et al, 2001]. In contrast, Primatone is an enzymatic digest of animal tissue. It is a complex mixture containing amino acids, oligopeptides, iron salts, lipids, and other trace low molecular weight substances [Schläger 1996].

In this study, two different thin film composite membranes were chosen according to previous characterization described in Chapter 4. The G-10 membrane, a loose nanofiltration membrane with a 2500 Da MWCO, and the HL membrane, with a 300-500 Da MWCO, were chosen. The effect of pH (expected charge difference at different pH‘s on membrane and for the peptides) and NaCl (affects interactions of peptides near the membrane) addition on the nanofiltration performance were investigated because these two parameters are expected to affect the actual separation process while changing the charge of the peptides and membrane or interacting with the membrane.

6.2 Experimental

6.2.1 Chemicals

Folin & ciocalteu's phenol reagent (FCR), o-phthaldialdehyde (OPA), β-mercaptoethanol, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox), sodium dodecyl sulfate (SDS), sodium carbonate, sodium hydroxide were purchased from Sigma (Oakville, ON, Canada). Yeast Extract (211929 lot#8219390) was purchased from BD Bioscience (Mississauga, ON, Canada) and phenylglycin and Primatone RL (2751016 lot# R24429) from MP Biomedicals (Montréal, QC, Canada). Sodium chloride and hydrochloric acid were bought from BDH (Mississauga, ON, Canada). The water used for all experiments was obtained from a Millipore system (Synergy® Ultrapure Water system, Millipore, Etobicoke, ON, Canada) with a conductivity of 0.056µS/cm.

6.2.2 Experimental Set-up

Nanofiltration experiments were carried out by investigating two different flat sheet thin film composite membranes which were previously characterized (Chapter 4) and both manufactured from GE Healthcare (Baie d'Urfe, Canada). The HL and G-10 membrane have a MWCO of 300-500Da and 2500Da respectively. The same experimental set-up as presented in Chapter 4 was used for the following nanofiltration.

6.2.3 Identification of TMP for Nanofiltrations

The appropriate TMP was identified from permeate flux measured at different pressures for 0.1%wt feed of yeast extract and Primatone. The filtrations were carried out at 24°C and at a flowrate of 1.8L/min.

6.2.4 Nanofiltration Experiments

Yeast extract and Primatone were dissolved in 2 L Millipore water as a 0.1 %wt solution. Membranes were used for 8 subsequent experiments (same feed source) and cleaned with 0.01M NaOH for 30 minutes after each experiment to obtain the same water flux as before the filtration. When not being used, the membrane was stored at room temperature in Millipore water. All experiments were carried out at a constant TMP of 2 MPa, a flowrate of 1.8 L/min, and at 24°C. The pH was either adjusted to 8 by 1 M NaOH or to 4 by 1 M HCl. For the experiments with NaCl, 150 mM NaCl was added. Permeate was collected until half of the

feed volume was filtered (VCR = 2). Both retentate and permeate fractions were stored at -20°C. The organic and inorganic content, the transmission of antioxidant capacity estimated by FCR assay, and the peptide concentration by OPA assay were determined for the the feed, retentate and permeate.

6.2.4.1 Experimental Design

In order to obtain reliable results the planning of experiments is essential. For experiments where more than one factor is to be investigated the use of a factorial design is advantageous as the significance of each factor is assessed for statistical significance by creating multiple linear regression models for each response parameter that is of interest. The filtration performance of yeast extract and Primatone was investigated with a duplicated 2^4 factorial design. The influence of the feed source, the membrane type, pH, and NaCl concentration on the filtration performance was assessed. Table 6.1 summarizes the low and a high conditions for each variable studied. For the pH an acidic and a basic condition was chosen because of the charge difference of the corresponding solution and membrane. The presence or absence of salt could influence the interaction of the molecules near the membrane and was therefore chosen as a factor to investigate. The filtration performance was characterized according to the total peptide transmission, the antioxidant capacity, the organic and inorganic content , and the average permeate flux.

Table 6.1 Experimental design - high and low level for each factor

Factor	High Level	Low Level
pH	8	4
NaCl [mM]	0	150
Feed Source	Yeast Extract	Primatone
Membrane	G-10	HL

6.2.5 Total solids, Organic, and Inorganic Compounds

The total solids content of the feed, retentate, and permeate fraction was obtained from the dry weight of a 10 mL sample contained in an aluminum dish and placed overnight in a 100°C oven (Hotpack, Waterloo, ON, Canada) as duplicates. The organic content represented the mass of solids burned after 10 minutes in a 550°C muffle furnace. The solid components still

present after the combustion in the muffle furnace were considered the inorganic content of the sample [Eaton et al., 1995].

6.2.6 Total peptide concentration assay (OPA)

The same method as presented in Chapter 5 was used for the determination of the total peptide concentration. A volume of 20 / 10 / 200 / 100µL of the feed, retentate, or permeate was added to 1mL OPA solution respectively for the analysis of the nanofiltration fractions.

6.2.7 Antioxidant capacity assay (FCR)

The same method as presented in Chapter 5 was used for the determination of the antioxidant capacity. A volume of 10µL of each sample (after freeze drying and dissolving at a concentration of 6mg total solids/mL) were used for the feed, retentate, and permeate of the nanofiltration fractions.

The antioxidant capacity was determined as the reducing capacity and obtained with the addition of the Folin & ciocalteu‘s reagent as the oxidant, which becomes reduced by the phenol ring present in the amino acids tyrosine, phenylalanine, and tryptophan. The colour change of the FCR by abstracting an electron from the antioxidant can be measured at 750nm [Huang et al., 2005].

6.2.8 Freeze drying

The same method as presented in Chapter 5 was used for the freeze drying of the samples.

6.2.9 Data Analysis

Significant factors affecting the filtration performance were identified by multiple linear regression using the software Design Expert® 6.1 (Stat-Ease, Minneapolis, USA). A factor was considered as significant if the p value was smaller than 0.05 with a corresponding confidence interval of 95%.

General linear regression models were created according to five characterization parameters: the total peptide transmission ratio, the organic content transmission ratio, the inorganic content transmission ratio, the average permeate flux [LMH], and the antioxidant capacity transmission ratio of each filtration. The models were created by a backward step-wise method

by keeping variables with $p<0.05$ only. Interactions between two, three or four factors were included in the model as well. No statistical outliers were present.

As an example for a 2^4 factorial design the multiple linear regression would yield an equation of the form:

$$Y = \alpha_0 + \alpha_1 x_1 + \alpha_2 x_2 + \alpha_3 x_3 + \alpha_4 x_4 + \alpha_5 x_1 x_2 + \alpha_6 x_1 x_3 + \alpha_7 x_1 x_4 + \alpha_8 x_2 x_3 + \alpha_9 x_2 x_4 + \alpha_{10} x_3 x_4 + \alpha_{11} x_1 x_2 x_3 + \alpha_{12} x_1 x_2 x_4 + \alpha_{13} x_1 x_3 x_4 + \alpha_{14} x_2 x_3 x_4 + \alpha_{15} x_1 x_2 x_3 x_4$$

Where: Y = dependent variable (filtration performance characterization parameters)

α = coefficient

x = independent variable (factors A, B)

6.3 Results and Discussion

6.3.1 Identification of TMP for Filtrations

The appropriate TMP was identified by performing a critical pressure profile, in total recycle operation, for the nanofiltration experiments with Primatone and yeast extract. As presented in Figure 6.1, different transmembrane pressures (TMP) were investigated for both feed sources. Similar to the ultrafiltration experiments, primatone as a feed source shows a higher flux at lower transmembrane pressures. For yeast extract, the permeate flux increases linearly with a TMP until 3 MPa and then starts to level off. Primatone on the other hand already levels off after a 2 MPa TMP and more significantly. Therefore it can be assumed that Primatone contains more molecules that can be attributed to fouling in the nanofiltration process. According to these results, a TMP of 2 MPa was selected for the factorial design for both types of feed, yeast extract and Primatone.

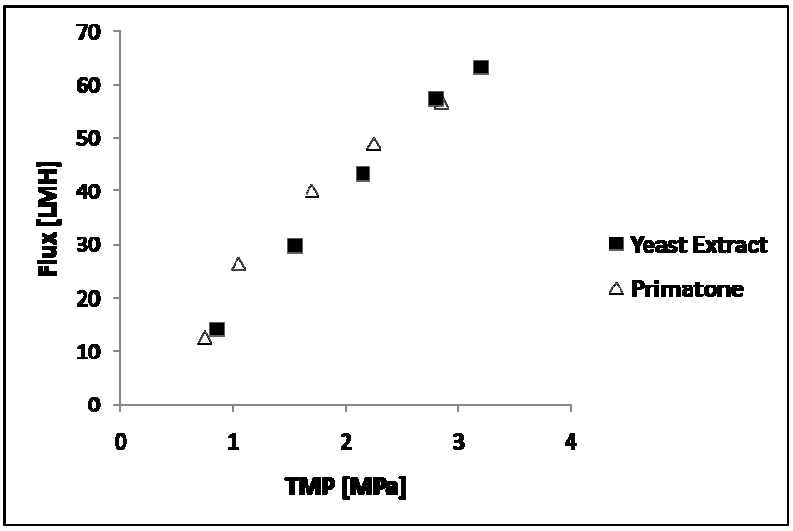

Figure 6.1 Critical pressure profile (total recycle opeation) for 0.1%wt yeast extract and Primatone

6.3.2 Filtration Performance of Yeast Extract and Primatone

The filtration performance of peptide mixtures was investigated with a 2^4 factorial design to evaluate on the effects of feed, membrane type, pH, and NaCl during nanofiltration concentration operations (continuous permeate removal). The total peptide transmission, the organic content transmission, the inorganic content transmission, the antioxidant capacity transmission, and the average permeate flux were selected as characterization (ie. filtration performance) parameters. The antioxidant activity of these fractions could be an interesting aspect on the bioactivity when tested in cell culture. The inorganic compound transmission is a parameter for the effect of salt transmission of each filtration. As yeast extract and Primatone are both protein hydrolysates obtained by an enzymatic hydrolysis of yeast cells or animal tissue respectively not all the organic compounds are peptides. Organic compounds are analyzed to determine a possible relationship to the peptide concentration which is important for the bioactivity testing in cell culture experiments.

6.3.2.1 Overall 2^4 factorial design

An overview of the 2^4 factorial design is presented in Figure 6.2.

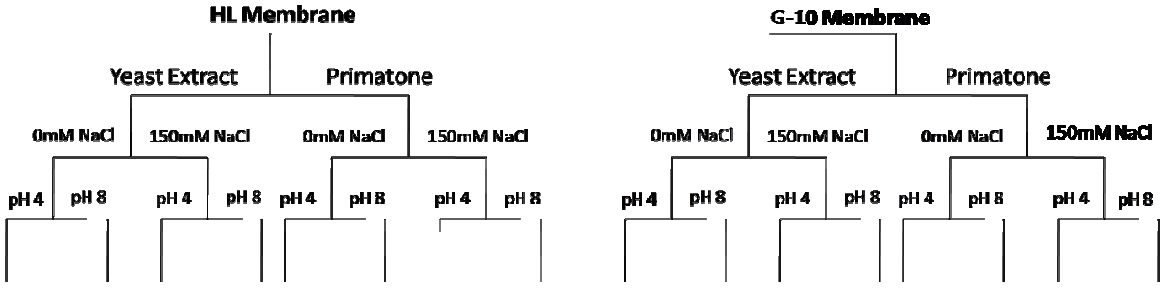

Figure 6.2 Overview of the 2^4 factorial design

For each performance parameter, a separate linear regression model was generated. Possible curvature of the different factors could be investigated after the factorial design has been completed to assure a linear relationship of these factors. The factors and interaction terms found to affect significantly the performance parameters ($p<0.05$) are listed in Table 6.2. The higher the coefficient of a specific parameter, the higher will be its significance on the characterization parameter. The membrane type shows the most significant influence for most of the performance parameters; except for the inorganic content transmission. The inorganic content represents essentially the mineral content of the sample and will reflect experiments where NaCl was added. None of the design variables were shown to significantly affect the antioxidant capacity transmission.

Table 6.2 Coefficients of the linear regression models of the overall factorial design with four factors A) Feed type B) Membrane type C) pH and D) NaCl and different characterization parameters

	A (Feed)	B (Membrane)	C (pH)	D (NaCl)	AB	AC	AD	BC
$T_{peptides}$	0.009	0.094	0.001	0.018	0.029		0.013	-0.025
$T_{organics}$	-0.049	0.15	-0.015		-0.034			-0.034
$T_{inorganics}$		0.005	-0.024	0.21				0.009
Perm flux	-1.71	-82.13	9.2	-5.12	-24.56	-1.57	-1.4	
$T_{antioxidant\ activity}$								

	BD	CD	ABC	ABD	ACD	BCD	ABCD
$T_{peptides}$	0.012			0.014			
$T_{organics}$							
$T_{inorganics}$	0.047	0.046				-0.04	
Perm flux	13.03	1.33			5.39		
$T_{antioxidant\ activity}$							

6.3.2.2 Factorial Design with investigation of feed source, pH and NaCl

To investigate the influence of factors other than the membrane, the design was divided according to membrane type with 3 factors, pH and NaCl and feed source (HL membrane in Table 6.3 and G-10 membrane in Table 6.4). As summarized in Table 6.3 and 6.4, the feed source affected significantly all the characterization parameters for both membranes except the antioxidant capacity transmission and the inorganic content. For the HL membrane, the feed source had the most significant impact on the average permeate flux, while the pH showed the most significant effect on the total peptide transmission and the organic content transmission. For the G-10 membrane, the feed source had the largest influence on the total peptide transmission, the organic content transmission, and the average permeate flux. For the HL membrane, the pH seems to be more important than the feed source of all response parameters. In summary, the feed source has a more significant effect for the G-10 membrane than for the HL membrane and the pH conditions affect the fractionation with the HL membrane.

Table 6.3 Coefficients of the linear regression models with three factors A) pH B) NaCl and C) Feed type and different characterization parameters for the HL membrane

	A (pH)	B (NaCl)	C (Feed)	AB	AC	BC	ABC
$T_{peptides}$	0.024	-0.02	-0.017				
$T_{organics}$	0.019		-0.015				
$T_{inorganics}$	-0.015	0.17	0.083				
Perm flux	11.4	-18.15	22.85	4.37	-0.88	-0.7	4.96
$T_{antioxidant\ activity}$							

Table 6.4 Coefficients of the linear regression models with three factors A) pH B) NaCl and C) Feed type and different characterization parametersfor the G-10 membrane

	A (pH)	B (NaCl)	C (Feed)	AB	AC	BC	ABC
$T_{peptides}$	-0.026	0.03	0.037			0.027	
$T_{organics}$			-0.083				
$T_{inorganics}$		0.26					
Perm flux	6.95	7.91	-26.28	-1.7	-2.26	-2.1	5.81
$T_{antioxidant\ activity}$							

6.3.2.3 Narrowed 2^2 Factorial Designs with investigation of pH and NaCl

As the feed source has a significant effect on most of the performance parameters for the G-10 and HL membranes, the factorial design was further reduced. In this case four different 2^2 factorial designs were obtained, one for each combination of feed source and membrane type. It is expected that a more detailed analysis of each 2^2 design will result in more information on the influence of pH and NaCl addition. As the transmission of antioxidant capacity was not shown to be significantly affected by any of the four factors studied, this parameter was omitted from this analysis.

The G-10 membrane with yeast extract, presented in Table 6,5, shows the significant effects of pH and NaCl addition for various performance parameters.

Table 6.5 Coefficients of the linear regression models with two factors A) pH and B) NaCl for different characterization parameters and the G-10 membrane with yeast extract

	A (pH)	B (NaCl)	AB
$T_{peptides}$	-0.02	0.057	-0.017
$T_{organics}$		0.062	
$T_{inorganics}$		0.23	
Perm flux	4.7	5.81	4.11

The linear regression model for the total peptide transmission suggests that both pH and NaCl addition have a significant impact on the filtration performance. The higher ionic strength resulting from the NaCl addition enhanced the total peptide transmission. Furthermore, the interaction term is significant for the total peptide transmission as shown in Figure 6.4A and 6.4B. The pH affected the total peptide transmission only when NaCl was added.

As seen in Figures 6.3C and 6.3D, the pH did not have a significant affect on the organic content transmission. In contrast, NaCl addition significantly affected the organic transmission content. As expected the inorganic content transmission, was related to the NaCl addition (Figures 6.3E and 6.3F). The average permeate flux with the G-10 membrane and yeast extract was affected by pH, NaCl addition and the interaction between these two factors (Figures 6.3 G and 6.3H). Similar to the total peptide transmission, the average permeate flux is affected by pH only when NaCl is added. The highest average permeate flux is obtained at high pH and with NaCl addition.

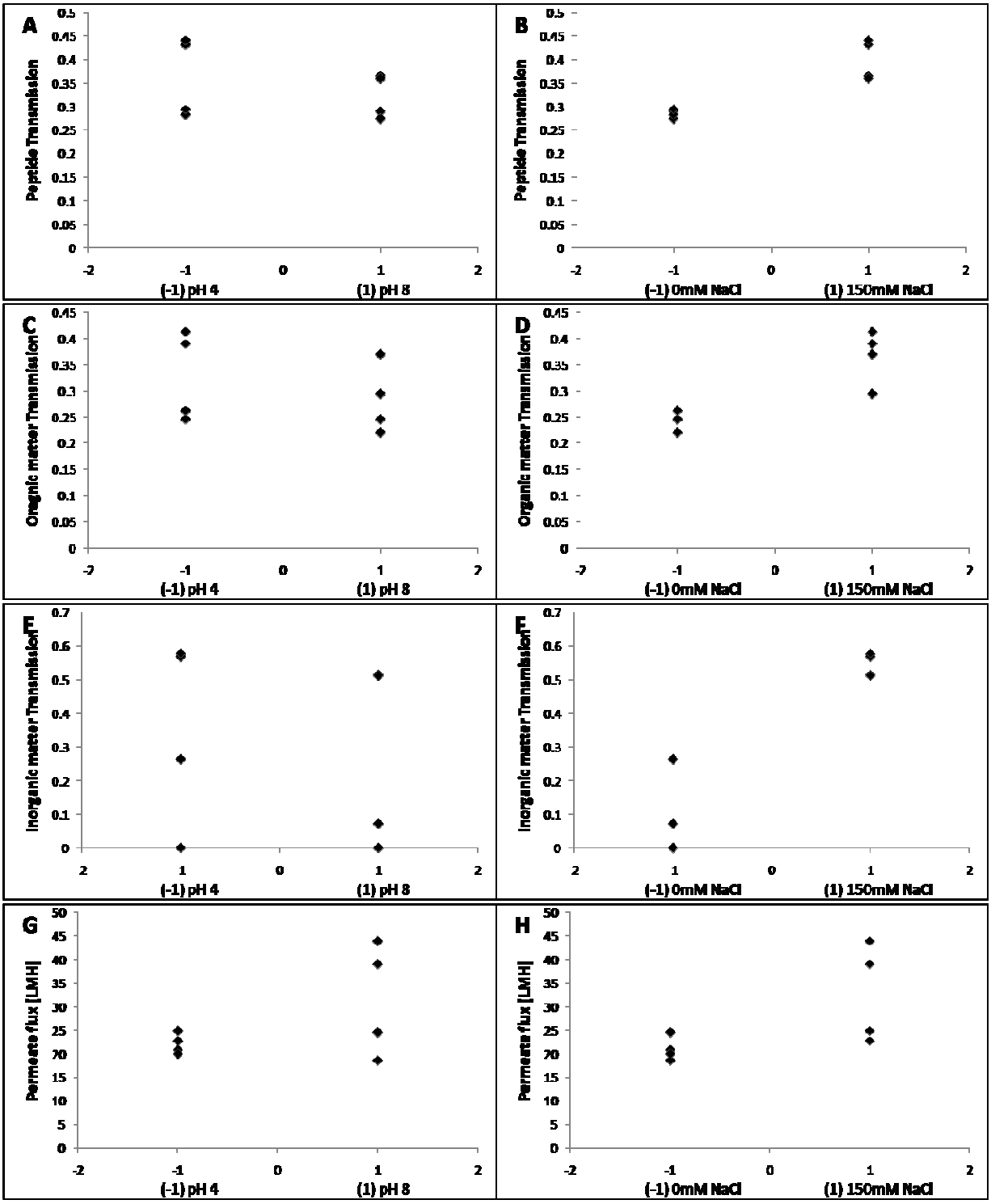

Figure 6.3 Effects of pH and NaCl on A) and B) total peptide transmission, C) and D) organic content transmission, E) and F) inorganic content transmission, and G) and H) Average Permeate Flux – experiments performed with yeast extract and the G-10 membrane

Table 6.6 summarizes the effects of pH and NaCl on the different performance parameters for yeast extract and the HL membrane.

Table 6.6 Coefficients of the linear regression models with two factors A) pH and B) NaCl, for different characterization parameters and the HL membrane with yeast extract

	A (pH)	B (NaCl)	AB
$T_{peptides}$			
$T_{organics}$	0.022		
$T_{inorganics}$	-0.061	0.13	0.13
Perm flux	10.56	-18.85	9.33

No significant effects were found for the total peptide transmission of peptides.

In contrast to the organic content transmission for the yeast extract fractionation with the G-10 membrane, the fractionation of yeast extract with the HL membrane was affected by the pH conditions.

The inorganic content transmission presented in Table 6.6, shows that the NaCl and the interaction term had the most significant effect, while the effect of pH was not as pronounced but still statistically significant. Taking into account that nanofiltration fractionation is based on charge and can be used to separate monovalent ions, the pH effect for the separation of ions is expected.

As for the fractionation of yeast extract with the G-10 membrane, pH, NaCl addition, and the interaction term of the two factors have a significant impact on the average permeate flux. The NaCl addition was shown to have the most significant effect on the average permeate flux, with the NaCl addition having a negative effect on the average permeate flux. Increasing the pH enhanced the organic content transmission. In general, a high pH and no NaCl addition had the highest average permeate flux, and the highest organic content transmission.

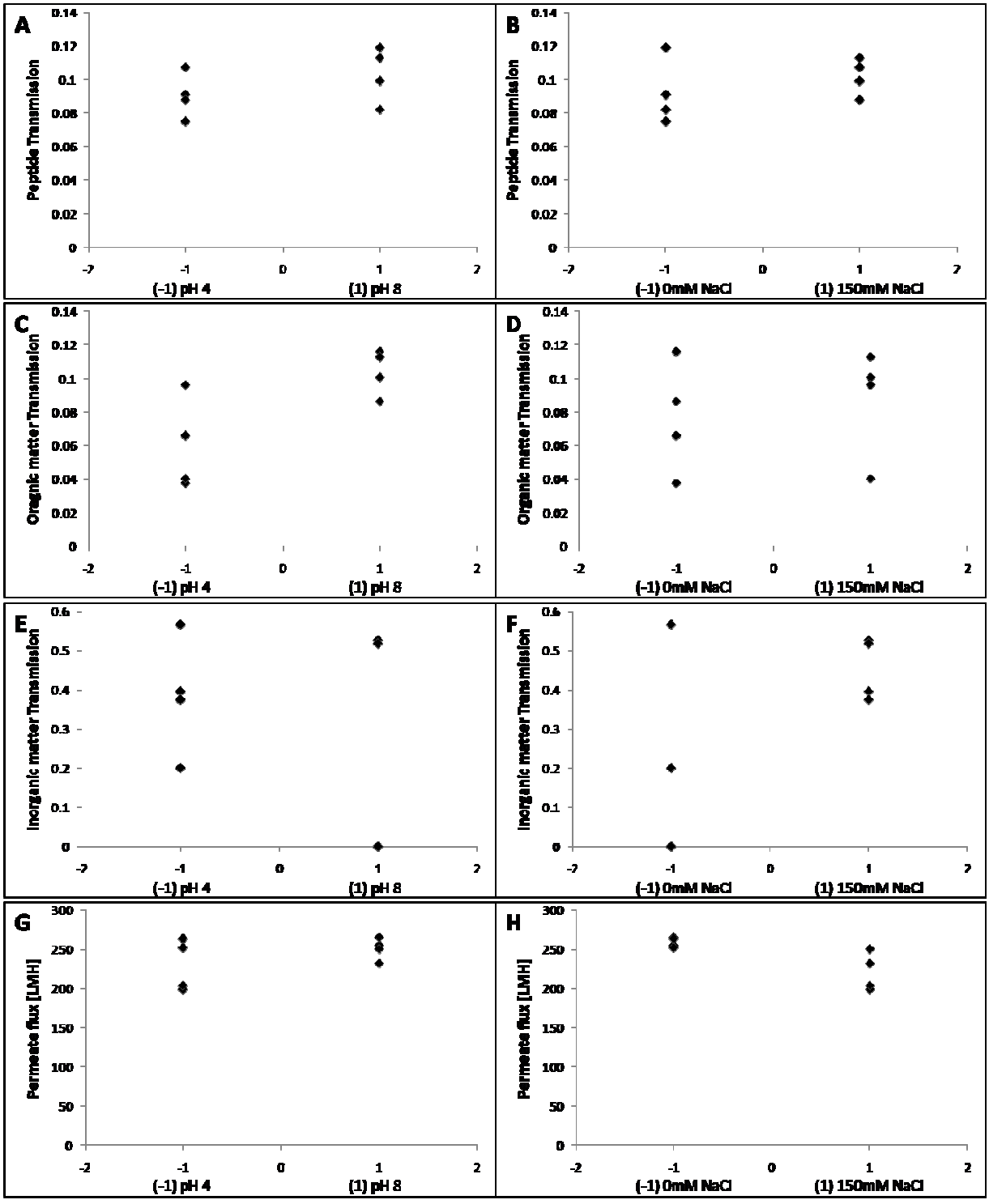

Figure 6.4 Effects of pH and NaCl on A) and B) total peptide transmission, C) and D) organic content transmission, E) and F) inorganic content transmission, and G) and H) Feed Flux – experiments performed with yeast extract and the HL membrane

The fractionation of Primatone with the G-10 membrane is summarized in Table 6.7 indicate fewer significant effects when compared to yeast extract.

Table 6.7 Coefficients of the linear regression models with two factors, A) pH and B) NaCl, for different characterization parameters and the G-10 membrane with Primatone

	A (pH)	B (NaCl)	AB
$T_{peptides}$	-0.031		
$T_{organics}$			
$T_{inorganics}$		0.29	
Perm flux	-9.21	10.02	7.51

The effect of pH and NaCl addition presented in Figures 6.5A and 6.5B, indicate that increasing pH results in a significant negative effect on the total peptide transmission. A low pH corresponds to a higher total peptide transmission.

The pH and NaCl addition had no significant effect on the oraganic content transmission.

The inorganic content transmission, as expected, was influenced significantly by NaCl addition.

For the average permeate flux, NaCl addition had the most significant influence. The pH and the interaction term also had some effect on the average permeate flux. The interaction term suggests that NaCl addition had an effect on the permeate flux at pH 4, but not at pH 8.

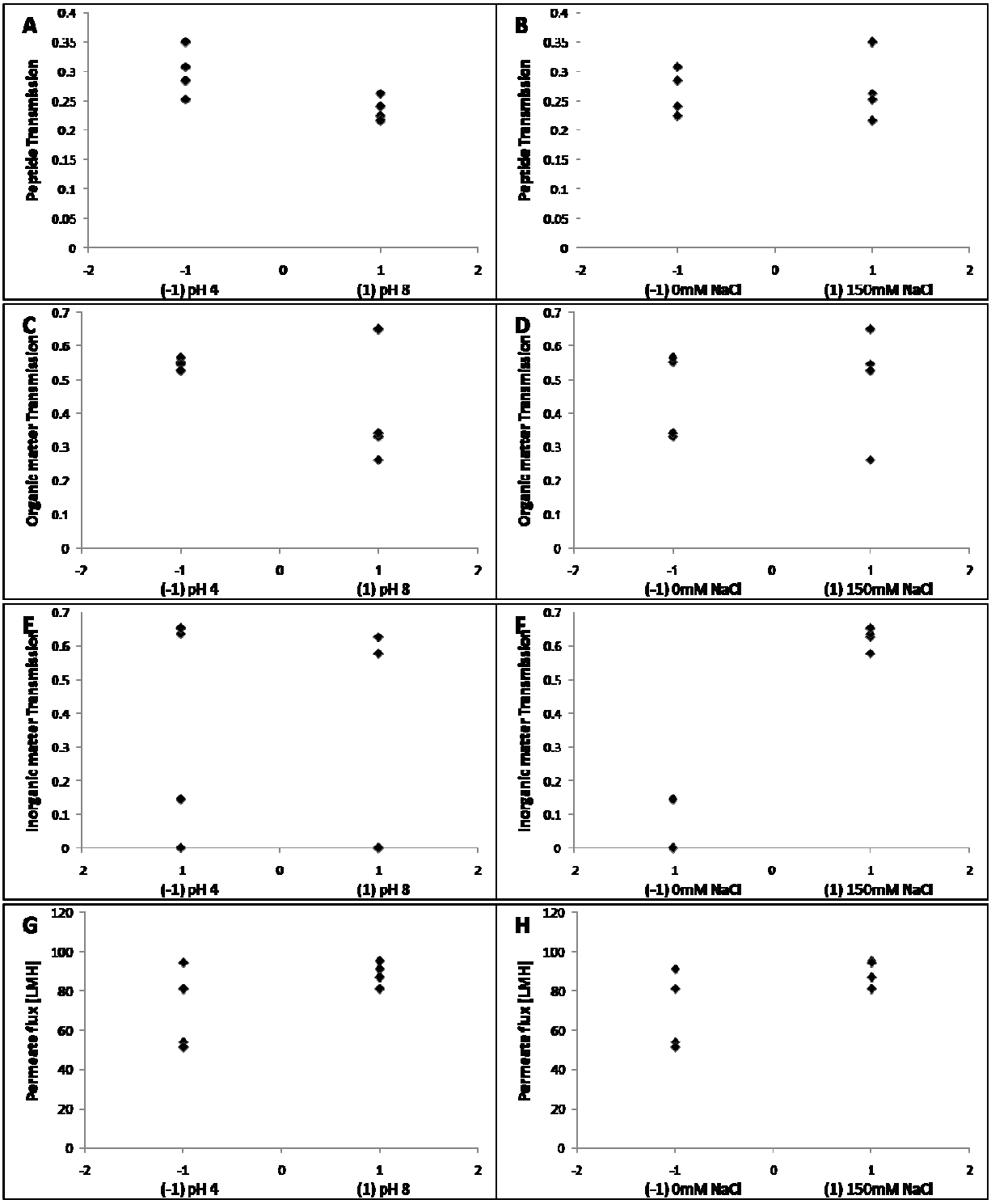

Figure 6.5 Effects of pH and NaCl on A) and B) total peptide transmission, C) and D) organic content transmission, E) and F) inorganic content transmission, and G) and H) Feed Flux – experiments performed with Primatone and the G-10 membrane

The fractionation of Primatone with the HL membrane, presented in Table 6.8, shows a large number of significant effects.

Table 6.8 Coefficients of the linear regression models with two factors, A) pH and B) NaCl for different characterization parameters and the HL membrane with Primatone

	A (pH)	B (NaCl)	AB
$T_{peptides}$	0.041		
$T_{organics}$	0.015	0.008	0.013
$T_{inorganics}$		0.2	
Perm flux	12.32	-17.45	

As seen in Figures 6.6A and 6.6B, only the pH had an effect on the total peptide transmission.

According to the organic content transmission, the higher the pH and NaCl addition, the higher transmission is achieved. Both pH and NaCl addition had a significant effect on the organic content transmission. Furthermore the interaction term is significant as well. The effect of NaCl addition is observed only at the high pH for the organic content transmission.

As for the G-10 membrane, only the NaCl addition influenced the inorganic content transmission. No effect of pH was observable.

The fractionation of Primatone with the HL membrane showed a significant effect of pH and NaCl addition. An increasing pH had a positive effect while the NaCl addition had a more pronounced negative impact for all factors except the inorganic content transmission.

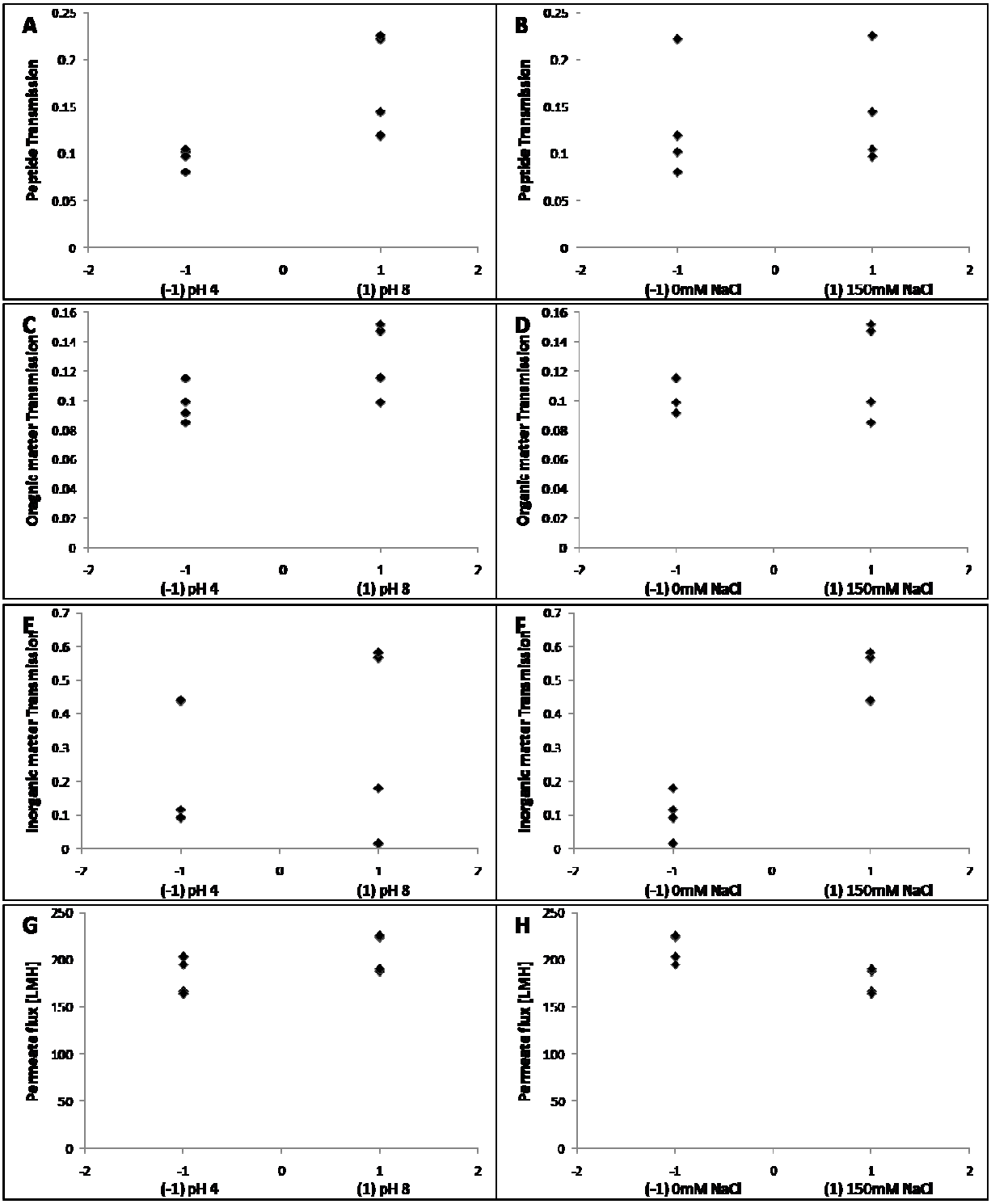

Figure 6.6 Effects of pH and NaCl on A) and B) total peptide transmission, C) and D) organic content transmission, E) and F) inorganic content transmission, and G) and H) Feed Flux – experiments performed with Primatone and the HL membrane

6.3.2.4 Nanofiltration fractionation - Summary

A summary of the significances (expressed as positive effect (+) or negative effect (-)) for pH and NaCl addition based on the corresponding coefficient and the different characterization parameters of the 2^2 factorial designs is given in Tables 6.9-6.12 for each combination of feed source and membrane type.

Comparing the total peptide transmission, the pH shows a significant effect for all feed source membrane combinations, except for yeast extract and the HL membrane. The NaCl addition showed an effect only for yeast extract and the G-10 membrane. Both feed sources fractionated with the G-10 membrane were negatively affected by the pH. This leads to the assumption that pH has a more important influence than the feed source. The interaction term was only significant for yeast extract and the G-10 membrane.

Table 6.9 Summary of the significance of pH, NaCl and interaction term for total peptide transmission of the 4 different 2^2 factorial design combinations (+ for positive effect / – for negative effect)

	pH $T_{peptide}$	**NaCl $T_{peptide}$**	**Interaction $T_{peptide}$**
YE G-10	-	+	-
YE HL			
Primatone G-10	-		
Primatone HL	+		

Looking at the different combinations of feed source and membrane type and the organic content transmission, it is obvious that the pH had a significantly positive effect for yeast extract and Primatone fractionation with the HL membrane which suggests a predominance of the pH compared to the feed source. The influence of NaCl addition was distinctive for yeast extract and the G-10 membrane and for Primatone and the HL membrane respectively suggesting an interaction between the membrane type and feed source since different membranes and feed source combinations were affected.

Table 6.10 Summary of the significance of pH, NaCl and interaction term for the organic content transmission of the 4 different 2^2 factorial design combinations (+ for positive effect / – for negative effect)

	pH $T_{organics}$	NaCl $T_{organics}$	Interaction $T_{organics}$
YE G-10		+	
YE HL	+		
Primatone G-10			
Primatone HL	+	+	+

The inorganic content reflects essentially the NaCl addition. But as shown in Table 6.11, pH also had a small impact on yeast extract when fractionated with the HL membrane. This shows that the nanofiltration mechanism is based on size and charge and that a change of pH could separate the ions differently. Inorganic components (ash) are present in yeast extract and Primatone and could also lead to a different distribution according to pH.

Table 6.11 Summary of the significance of pH, NaCl and interaction term for the inorganic content transmission of the 4 different 2^2 factorial design combinations (+ for positive effect / – for negative effect)

	pH $T_{inorganics}$	NaCl $T_{inorganics}$	Interaction $T_{inorganics}$
YE G-10		+	
YE HL	-	+	+
Primatone G-10		+	
Primatone HL		+	

Table 6.12 shows that the average permeate flux was affected by both pH and NaCl addition. In general when using the HL membrane, pH had a positive effect and NaCl addition had a negative effect on the average permeate flux. Regarding the G-10 membrane, the feed source influenced the average permeate flux, with an increasing pH having a positive impact for yeast extract and a negative impact for Primatone. The NaCl addition on the other hand had a significantly positive effect for both feed sources.

Even though the two membranes are thin film composite materials, different nanofiltration performances were obtained, which proves that the different membrane surface charge and MWCO can alter according to pH and NaCl addition.

Table 6.12 Summary of the significance of pH, NaCl and interaction term for the average permeate flux of the 4 different 2^2 factorial design combinations (+ for positive effect / – for negative effect)

	pH Flux	NaCl Flux	Interaction Flux
YE G-10	+	+	+
YE HL	+	-	+
Primatone G-10	-	+	+
Primatone HL	+	-	

The antioxidant capacity transmission was not significantly affected by pH and NaCl addition for any of the feed source and membrane combination. This is assumed to be due to a large standard deviation in the duplicates.

Through this factorial design, the importance of pH and NaCl addition and their interaction were investigated. Looking at the detailed experimental estimates and their corresponding standard deviation summarized in Table 6.13 for the total peptide transmission, the organic content transmission, inorganic content transmission, antioxidant capacity transmission, and the average permeate flux, provides information on their reproducibility.

Table 6.13 Summarized results of the total peptide transmission ($T_{peptide}$), organic content transmission ($T_{organics}$), inorganic content transmission ($T_{inorganics}$), and the average permeate flux (J_f) (n=2)

Membrane	Feed	pH	NaCl	$T_{peptide}$	$T_{organics}$	$T_{inorganics}$	J_f [LMH]	$T_{antioxidant\ activity}$
G-10	YE	8	-	0.28 ± 0.01	0.23 ± 0.02	0.03 ± 0.05	21.6 ± 4.2	0.49 ± 0.26
G-10	YE	8	+	0.36 ± 0.01	0.33 ± 0.05	0.51 ± 0.01	41.5 ± 3.5	0.48 ± 0.04
G-10	YE	4	-	0.29 ± 0.01	0.25 ± 0.01	0.16 ± 0.01	20.4 ± 0.6	0.78 ± 0.07
G-10	YE	4	+	0.44 ± 0.01	0.40 ± 0.02	0.57 ± 0.01	23.8 ±1.5	0.54 ± 0.26
G-10	Prim*	8	-	0.23 ± 0.01	0.34 ±0.01	0	86.1 ± 7.0	0.68 ± 0.13
G-10	Prim*	8	+	0.24 ± 0.03	0.44 ± 0.27	0.60 ± 0.03	91.1 ± 5.9	0.60 ± 0.12
G-10	Prim*	4	-	0.30 ± 0.01	0.56 ± 0.01	0.07 ± 0.01	52.7 ± 1.9	0.66 ± 0.01
G-10	Prim*	4	+	0.30 ± 0.07	0.54 ± 0.01	0.64 ± 0.01	87.7 ± 9.5	0.58 ± 0.24
HL	YE	8	-	0.10 ± 0.02	0.10 ± 0.02	0	260.3 ± 7.1	0.45 ± 0.05
HL	YE	8	+	0.11 ± 0.01	0.11 ± 0.01	0.52 ± 0.01	241.3 ± 13.1	0.73 ± 0.24
HL	YE	4	-	0.08 ± 0.01	0.05 ± 0.02	0.36 ± 0.26	257.9 ± 8.1	0.61 ± 0.11
HL	YE	4	+	0.10 ±0.01	0.07 ± 0.04	0.38 ± 0.01	201.5 ± 3.5	0.46 ± 0.18
HL	Prim*	8	-	0.16 ± 0.07	0.11 ± 0.01	0.08 ± 0.03	224.9 ± 0.9	0.51 ± 0.19
HL	Prim*	8	+	0.18 ± 0.05	0.15 ± 0.01	0.57 ± 0.01	188.8 ± 1.2	0.44 ± 0.30
HL	Prim*	4	-	0.09 ± 0.01	0.10 ± 0.02	0.10 ± 0.02	198.9 ± 5.5	0.33 ± 0.02
HL	Prim*	4	+	0.10 ± 0.01	0.09 ± 0.01	0.44 ± 0.01	165.3 ± 1.4	0.61 ± 0.36

* Primatone

The HL membrane has much higher average permeate flux than the G-10 membrane as shown in Figure 6.7 - comparing all the different conditions with different membranes, feed sources, pH, and NaCl addition. This finding does not agree with the G-10 membrane known as a loose nanofiltration membrane with a reported 2500 Da MWCO, significantly higher than the 300-500Da MWCO reported for the HL membrane. Both membranes are based on thin film composite material, but the surface charge of both membranes are very different as characterized by the zeta potential (Chapter 4). According to their charge, different interactions are expected between the molecules and the membrane surface and pores which would be associated with different filtration behaviour as presented in Figure 6.7.

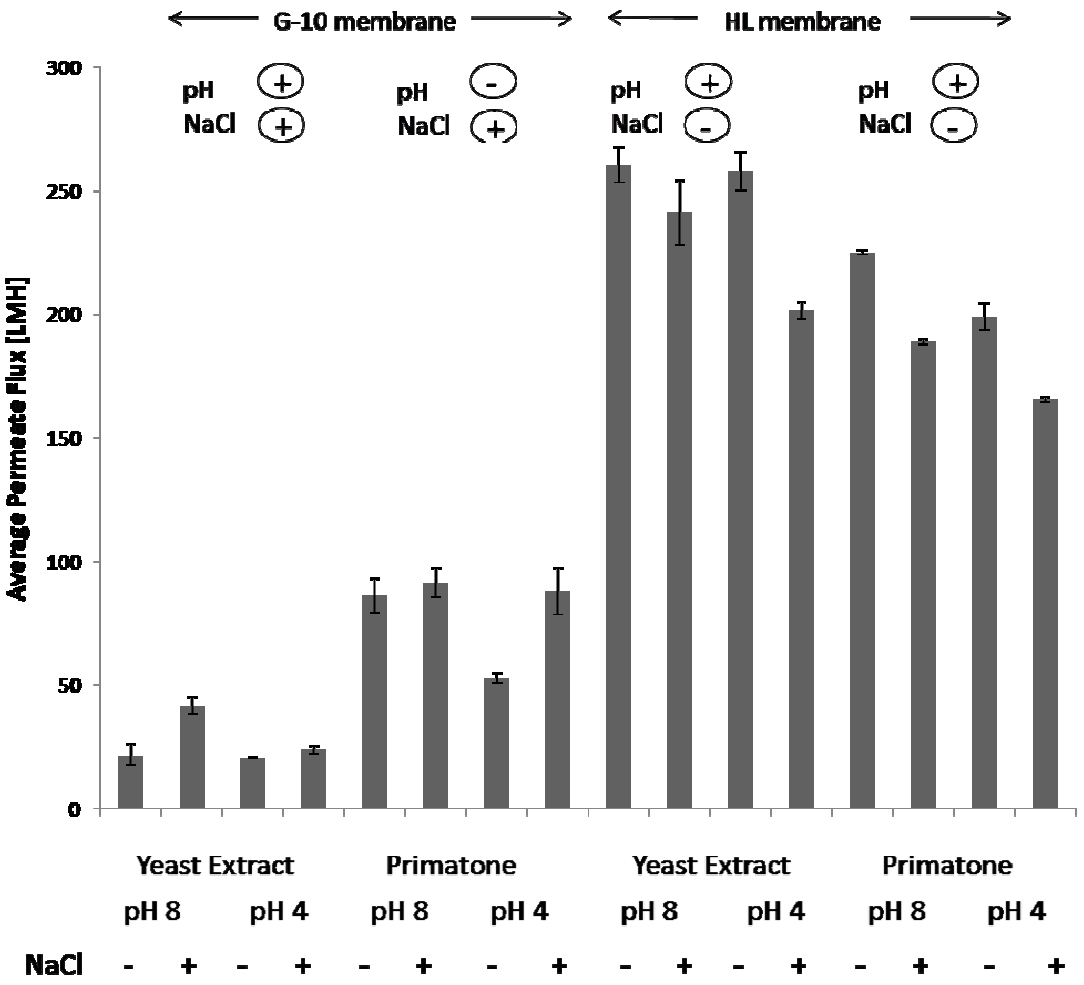

Figure 6.7 Summary of the feed flux for all experimental conditions investigated in this study (n = 2). The positive or negative sign on top of the graph shows the effect of pH and NaCl on the filtration performance regarding the feed flux in the specific factorial design.

In addition to the average permeate flux, the evolution with time of the permeate flux is different according to membrane type and would suggest different fouling behaviour (Figure 6.8). Whereas the permeate flux of the HL membrane gradually decreased during the entire filtration, the permeate flux of the G-10 membrane has an intial sharp decrease followed by a more gradual decrease. These two different permeate flux behaviour highlight the different fouling attributes of the two membranes. As the G-10 membrane has a 2500Da MWCO, it could theoretically be classified as an ultrafiltration membrane and therefore it is not farfetched to see a similar two phase fouling behaviour like ultrafiltration membranes. According to the contact angle measurement of the two membranes, the G-10 membrane should demonstrate lower fouling behaviour as it shows a higher hydrophilic character [Koehler et al., 2000]. But

the fouling behaviour is not only dependent on the membrane pore size and hydrophobicity but also on the feed composition as the different charged molecules have an influence on the interaction to the charged membrane. Therefore also the zeta potential of the membrane has an influence which is very different from the two membranes.

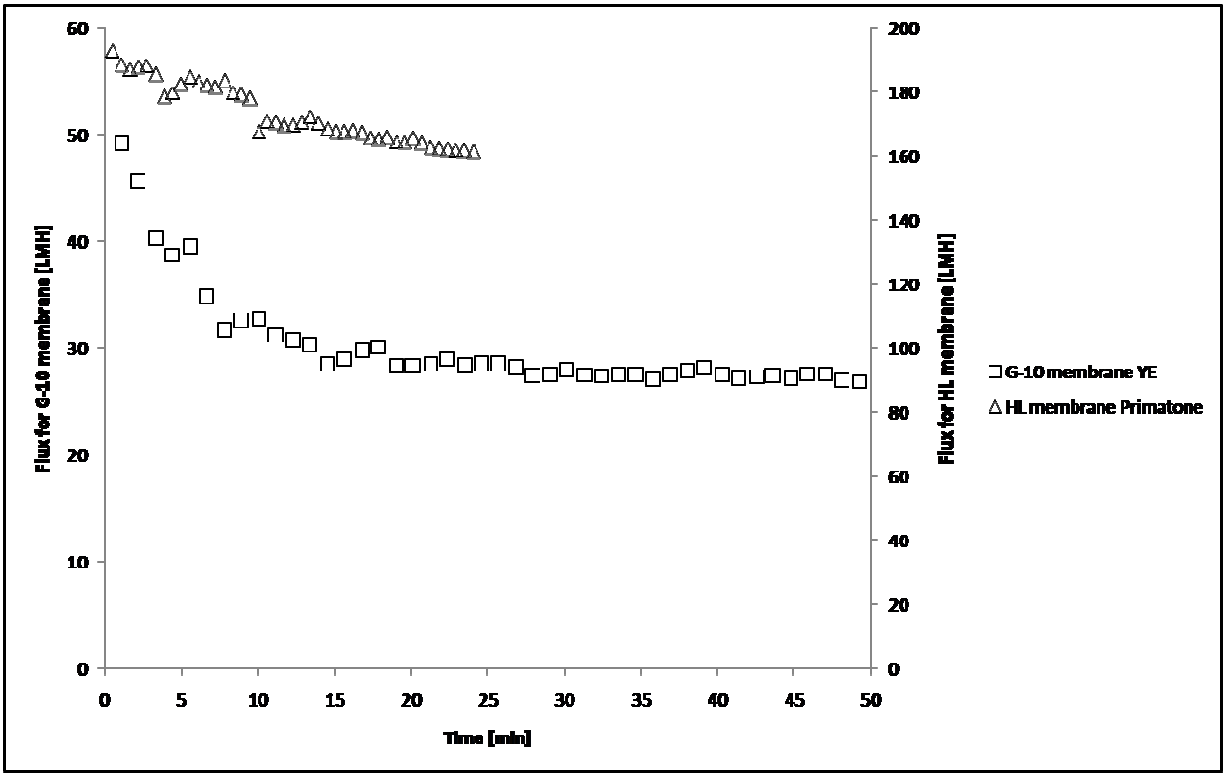

Figure 6.8 Permeate flux profile of the G-10 and HL membrane at pH 4 with NaCl and yeast extract or Primatone?

Comparing the total peptide transmission and the organic content transmission summarized in Figures 6.9 and 6.10 for both feed sources, a higher transmission is observed for the G-10 membrane compared to the HL membrane. The NaCl addition to the feed increased the total peptide and the organic content transmission for yeast extract. For Primatone, the increase was only observed at pH 8. The NaCl addition at pH 4 decreased the total peptide and organic content transmission. When comparing the average permeate flux to the total peptide transmission, the lower the permeate flux the higher the total peptide transmission.

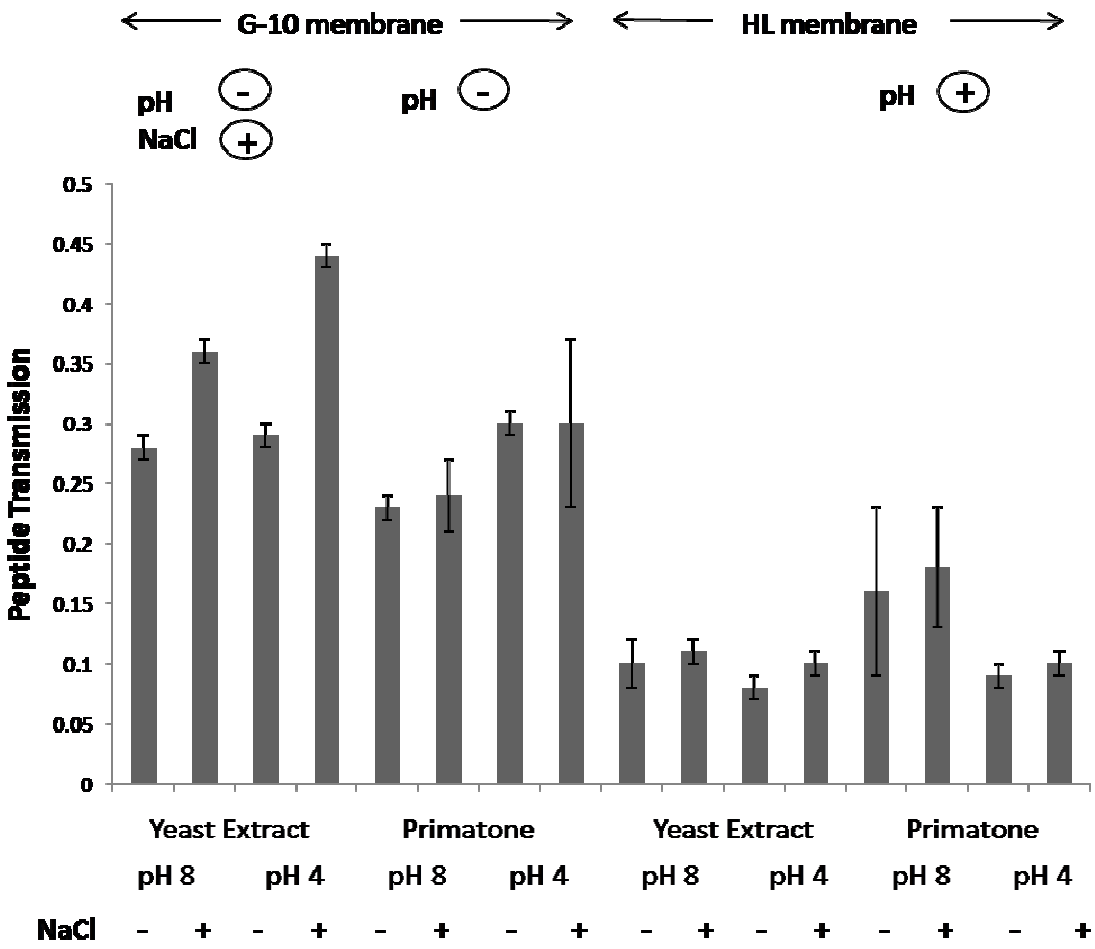

Figure 6.9 Summary of the total peptide transmission for all experimental conditions investigated in this study. The positive or negative sign on top of the graph shows the effect of pH and NaCl on the filtration performance regarding the total peptide transmission in the specific factorial design.

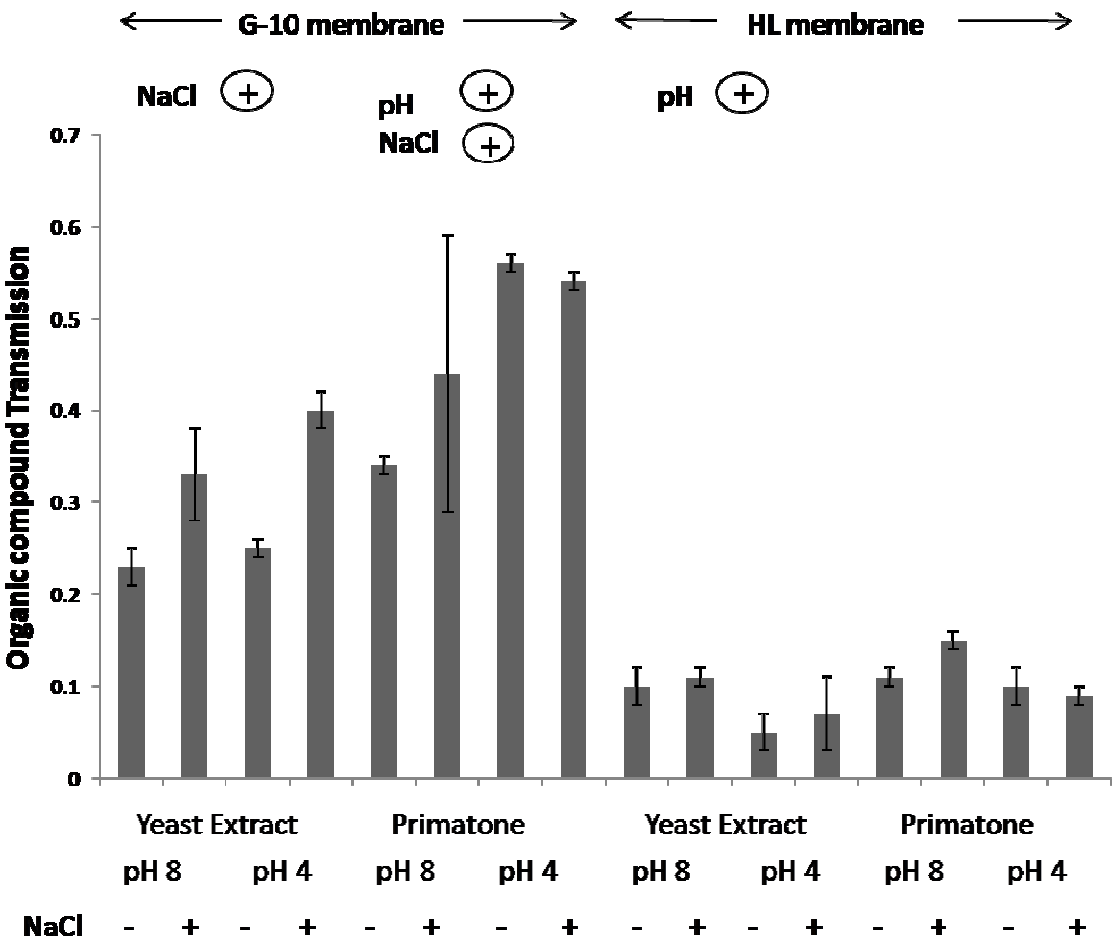

Figure 6.10 Summary of the organic content transmission for all experimental conditions investigated in this study. The positive or negative sign on top of the graph shows the effect of pH and NaCl on the filtration performance regarding the organic content transmission in the specific factorial design

Comparing the transmission of inorganic content, it was expected and could be shown that the NaCl had the highest impact on the distribution (no graph shown, see Table 6.13). But independent of the feed source, the distribution of the inorganic content transmission in the experiments with added NaCl was higher at pH 4 for the G-10 membrane and higher at pH 8 for the HL membrane. As nanofiltration membrane separates according to charge and size of molecules, the pH is a dominant factor for the separation of monovalent ions (NaCl) and might be a significant factor for the two tested membranes.

According to the literature 0-70% of sodium chloride is often rejected in nanofiltration membranes [Schäfer et al., 2005]. This was observed in this study for all the experiments with NaCl addition.

As presented in Figure 6.11, the antioxidant capacity transmission displayed very poor reproducibility with high standard deviations for most samples. It is possible that the nanofiltration does not selectively transmit or reject peptides with antioxidant activity; therefore it would be difficult to reproduce an experiment to obtain the same result. But already presented in the ultrafiltration separation process (Chapter 5) the antioxidant peptides could not be concentrated in the permeate fraction. This would have been expected as the smaller peptides (between 2 and 16 amino acid residues) obtain mostly antioxidant activity [Chen et al., 1996, Moure et al., 2006]. The measurement of antioxidant capacity is a sensitive method as it is very reactive with the measurement of reducing capacity. The problem is most likely attributed to the small concentration of antioxidant capacity present in the samples which would suggest a too low sensitivity of the method.

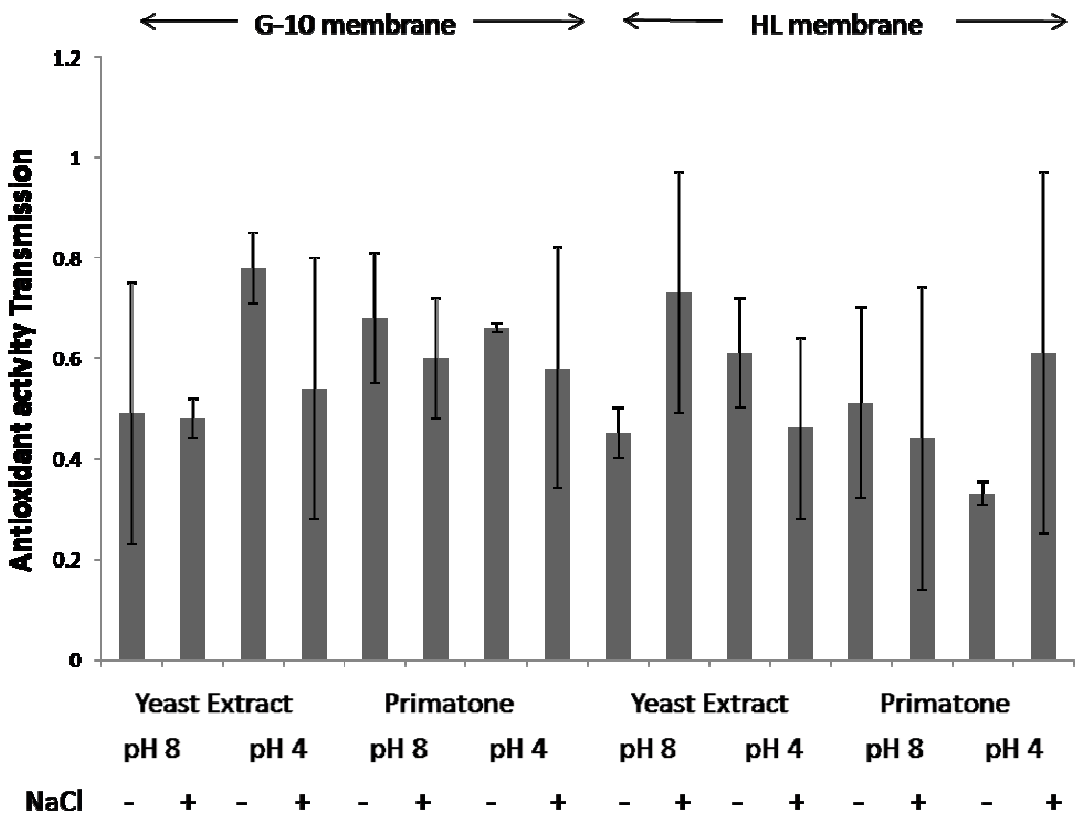

Figure 6.11 Summary of the antioxidant capacity transmission for all experimental conditions investigated in this study

It can be concluded that both feed sources are of different composition and that the membrane type makes an important difference for the filtration behaviour. The pH and the NaCl addition also showed significant effects in the distribution of total peptides, organic content and inorganic content in the permeate and the retentate fractions.

Different conditions (pH and ionic strength) for the nanofiltration of other protein hydrolysates were investigated in studies by different authors [Pouliot et al., 1999 / Groleau et al., 2004 / Tessier et al., 2006]. The studies performed with different pH and ionic strength also suggest that the pH and NaCl have different effects on the filtration performance on the separation on protein hydrolysates. Furthermore, for different membranes different effects of pH and NaCl were found. The results obtained in the presented study can not be compared with the studies from the literature, as the protein hydrolysate, system set-up, operating conditions, and the membranes are not identical.

6.4 Conclusions

Performing the 2^4 factorial design provided inofrmation on the effect of NaCl addition and pH for the fractionation of yeast extract and Primatone as feed sources and two different nanofiltration membranes. Multiple linear regression models were developed for five independent filtration characterization parameters, total peptide transmission, organic content transmission, inorganic content transmission, average permeate flux and antioxidant capacity transmission. By breaking down the overall factorial design in smaller 2^2 experimental designs, pH and NaCl addition showed different impacts according to the feed source and membrane combination.

A significant difference between the two membranes was observed for the total peptide transmission with around 10% for the HL membrane and around 30% for the G-10 membrane for both feed sources. Relating this total peptide transmission to the average permeate flux it was shown that a lower permeate flux leads to a higher total peptide transmission. Although the G-10 membrane has a reported higher MWCO than the HL membrane, it showed a lower average permeate flux which was independent of the feed source.

Considering that the two membranes had different membrane surface characteristics, it can be suspected that the charge of the membrane resulted in different interactions with the peptides in the feed and therefore affected their transmission ratios and the associated permeate flux.

The average permeate flux was affected the most significantly by pH and NaCl addition for all feed source membrane combinations. Therefore the average permeate flux seems to be dependent on the charge and the ionic strength of a solution.

Furthermore the standard deviations for the characterization methods were as expected and demonstrated good reproducibility. The measurement of the transmission of antioxidant capacity did not show any significant effect due to high variations in the replicates. But the high variations can be attributed to a low concentration of antioxidant capacity present in the sample and therefore a too low sensitivity of the method.

Further work should investigate the influence of pressure, temperature, and crossflow velocity on the filtration performance on peptide separation. Futhermore, the investigation of other membranes should be considered. The effect of higher and lower pH and NaCl concentrations would indicate whether the effect of pH and NaCl are linear functions.

Finally, the analysis of basic, neutral, and acidic peptides would be helpful in determining the filtration behaviour of different membranes at different pH and different salt concentrations with regard to peptide selectivity. A specific deterimination of individual peptides present in the permeate and retentate fractions could be achieved by HPLC-MS. Knowing which peptides are present in the feed source and after the fractionation by nanofiltration would be helpful in determining peptides which show an enhancing effect in serum-free cell culture.

Nanofiltration is an interesting and effective method to separate peptides. As different membranes show different results the membrane is a critical parameter of the system and could be changed to obtain the expected fractionation.

Chapter 7: Analysis by RP-HPLC of peptides from yeast extract and Primatone

Overview

The analysis of a peptide mixture is a challenge as peptides are similar in size; therefore a sensitive method has to be chosen for the analysis. Analysis of peptides by LC-MS provides a fingerprint of the individual peptides present in the mixture, but is limited when there are many individual peptides and is expensive and difficult to maintain. Reversed-phase high pressure liquid chromatography (RP-HPLC) represents an alternative and less expensive method which separates molecules according to their hydrophobicity. For the analysis of the fractions obtained from the nanofiltration experiments, RP-HPLC was used in combination with a pre-column phenyl-isothiocyanate (PITC) derivatization step before separating the samples on an Octadecyl-2PW column. Although it is believed that incomplete separation of the peptide mixture was achieved and the molecular weight of the different peptides could not be obtained, differences were observed for some of the fractions. Visualized by principle component analysis, the fractions from the ultrafiltration step showed different peptide profiles when compared to the fractions from the nanofiltration operation. It could be observed that the samples of both feed sources obtained from the ultrafiltration pretreatment were different from the samples obtained by nanofiltration. RP-HPLC combined with principal component analysis can provide information of the peptide profile of a peptide mixture.

7.1 Introduction

High pressure liquid chromatography (HPLC) is widely used for the analysis of proteins and peptides. Reversed phase HPLC separates protein or peptide mixtures according to their relative hydrophobicity. This method consists of a non-polar (hydrophobic) column and a moderately polar (hydrophilic) mobile phase [Horst, 2006]. Due to the hydrophobicity of the column packing material, the more hydrophobic molecules will have stronger interactions with the stationary phase and therefore result in a longer retention time [Dong, 2006].

RP-HPLC is often the method of choice for the analysis of amino acids or peptides because of the ability to separate the different amino acids according to their different hydrophobic character [Horst, 2006 / Dong, 2006]. The separation according to their size would be difficult as the size of amino acids and peptides are similar. One limitation is that only four amino acids, phenylalanine, histidine, tryptophan, and tyrosine possess a chromophoric group which can be detected by UV.

The visualization of peptides can be improved by derivatization with a chromophoric compound added to free N-termini. There are different derivatization methods available, and can be applied as a pre-column or post-column derivatization. A post-column derivatization is the derivatization after the separation process but entering the detector; therefore a reaction chamber in which the derivatization can occur needs to be added in the HPLC system set-up. The pre-column derivatization is performed before the injection in the system and has therefore the advantage that the chromatographic system does not have to be changed, but is a time intensive procedure [Nollet, 2000].

Different reagents such as OPA or phenyl isothiocyanate （PITC) are commonly used to derivatize amino acids. OPA has the disadvantages of being less sensitive, the derivatives are unstable and only primary amines are derivatized [Siebert et al., 1991]; therefore proline and hydroxyproline are undetectable because they only contain secondary amines [Cooper et al., 2001]. PITC on the other hand allows the derivatization of primary and secondary amines and produces stable and reproducible derivatives [Cohen et al., 1986, Colilla et al., 1991].

In this chapter, the analysis of peptides from yeast extract and Primatone fractionated by nanofiltration was investigated in more detail by RP-HPLC. The total peptide quantified by the OPA assay (Chapters 5 and 6) is limited and can not distinguish individual peptides. The analysis by RP-HPLC should be able to identify differences in the peptide profile of the nanofiltration fractions produced with different membrane feed source, pH, or NaCl addition conditions.

7.2 Experimental

7.2.1 Chemicals

Phenyl-isothiocyanate (PITC), amino acid standard (AAS18), tryptophan, glutamine, asparagine and sodium acetate were purchased from Sigma (Oakville, ON, Canada). Yeast Extract and Primatone were both purchased from BD Bioscience (Mississauga, ON, Canada). Triethylamine (TEA), acetonitrile, and glacial acetic acid were purchased from Fluka (Oakville, ON, Canada). All chemicals were of HPLC grade. The water for all experiments was obtained from a Millipore system (Synergy® Ultrapure Water system, Millipore, Etobicoke, ON, Canada) with a conductivity of 0.056μS/cm.

7.2.2 Derivatization of samples

All the fractions collected from the ultrafiltration and nanofiltration experiments were derivatized. A 20 μL of each freeze dried sample (dissolved at a concentration of 6mg total solids/mL) was vacuum dried (National Appliance Company, Memphis, TN, USA). As a coupling agent, the addition of 20 μL of a solution containing ethanol:water:triethylamine in a 2:2:1 ratio (for example 2 parts of ethanol (20 μL), 2 parts water (20 μL), and 1 part TEA (10μL)) was added to each sample and vacuum dried again. For the derivatization, a solution containing ethanol:water:triethylamine:PITC in a 7:1:1:1 (volumetric ratio) was added in a nitrogen environment and incubated for 20 min in a dark environment. After the incubation period, the samples were vacuum dried again and dissolved in 100 μL 50 mM sodium acetate buffer at pH 6.35 and containing 0.4 mL/L TEA.

The amino acid standard, containing 20 different amino acids, was prepared with the amino acid standard suite purchased from Sigma and three additional amino acids (tyrosine,

tryptophan and glutamine), according to the same procedure as described above, with the exception that only 10 μL of each (amino acid standard, tyrosine, tryptophan, or glutamine) was used. The three individual amino acids were first dissolved in water as a 2.5 μM solution. After the derivatization and drying, the amino acid standard and the three individual amino acids were each solubilised in 125 μL of the 50 mM sodium acetate buffer at pH 6.35 and containing 0.4 mL/L TEA and subsequently combined. The above methods were adapted from Cooper et al. (2001).

7.2.3 RP-HPLC

The chromatography was carried out on a Varian Pro Star HPLC system with an Octadecyl-2PW column and an Octadecyl-2PW guard column (Tosoh Bioscience, Montgomeryville, PA, USA). The analysis was performed at 40°C with 50 mM sodium acetate at pH 6.35 and containing 0.4 mL/L TEA as solvent A and 60% acetonitrile as solvent B. The gradient was optimized as follows: 0% B at 0min, 0-10% B for 5 min, 10-12% B for 3 min, 12-14% B for 4 min, 14-20% B for 6 min, 20-21% B for 3 min, 21-34% B for 0.5 min, 34-45% B for 1.5 min, 45-50% B for 2 min, 50-52% B for 0.5 min, 52-55% B for 1.5 min, 55-56% B for 3 min, 56-100% B for 0.5 min, 8.5 min at 100% B, 10min at 0% B for equilibration. The flowrate was 1 mL/min and the detection measured at 254 nm.

7.2.4 Data Analysis

For each sample, the height of every peak with an absorption higher than 10 mAU was considered for analysis. The peaks considered for analysis are summarized in Table 7.1.

The height of every peak in a given sample was collected and analyzed by principal component analysis (PCA) of the covariances using STATISTICA 8.0 (StatSoft, Tulsa, OK, USA). In PCA, the dimensionality of the data set is reduced by extracting an orthogonal set of principal components (PCs) made up of linear subsets of the original ordinates; the extraction is designed so that the maximum amount of variance is concentrated in the first PC, with the second largest amount of variance contained in the second PC, etc. [Legendre and Legendre, 1998; Weber and Legge, 2009].

Table 7.1 Peaks and their corresponding retention times considered for analysis

Peak No.	Ret time [min]	Peak No.	Ret time [min]	Peak No.	Ret time [min]
1	1.66	14	9.64	27	24.20
2	1.91	15	11.00	28	24.50
3	2.21	16	11.57	29	24.90
4	2.40	17	15.57	30	25.90
5	2.64	18	15.84	31	26.90
6	2.91	19	20.56	32	29.10
7	3.15	20	21.20	33	29.40
8	3.50	21	21.52	34	29.65
9	4.10	22	21.93	35	30.46
10	4.80	23	22.20	36	30.50
11	5.59	24	22.50	37	30.92
12	7.20	25	23.52	38	33.22
13	7.95	26	23.80	39	33.90

The data was subjected to a natural logarithm transformation as described by Weber and Legge, (2009), prior to ordination. This transformation improved the data restrictions associated with PCA. Principle component analysis was performed using correlations. The peaks not present in a given sample were coded as 0 mAU.

7.3 Results and Discussion

7.3.1 RP-HPLC Chromatograms

The chromatogram of the derivatized amino acid standard containing the 20 different amino acids and separated by RP-HPLC is shown in Figure 7.1. The 20 different amino acids were separated according to their hydrophobicity, with the more hydrophilic amino acids eluting first [Dong, 2006]. The amino acid standard was used as a measure for the quality of the separation and for comparison with yeast extract and Primatone that contain free amino acids, peptides, and proteins. Since the separation by RP-HPLC is according to hydrophobicity and not size, it is possible that a peptide containing a few amino acids will show the same or a similar hydrophobicity than a single amino acid and will elute at the same retention time. A few peptides can also have the same elution time if they possess a similar hydrophobicity.

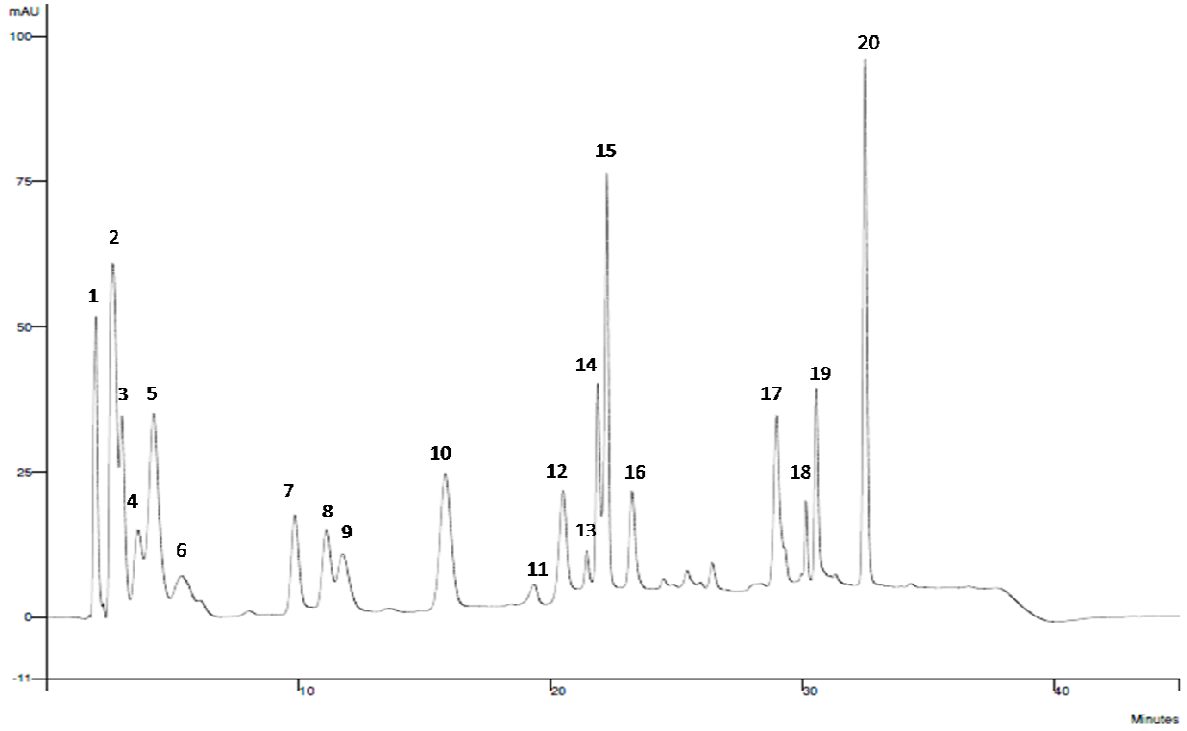

Figure 7.1 Chromatogram of the amino acid standard showing 1) Aspartic acid 2) Glutamic acid 3) Asparagine 4) Serine 5) Glutamine 6) Glycine 7) Histidine 8) Arginine 9) Threonine 10) Alanine 11) Proline 12) Tyrosine 13) Valine 14) Methionine 15) Cysteine 16) Isoleucine 17) Leucine 18) Phenylalanine 19) Tryptophan 20) Lysine

In Figure 7.2, the RP-HPLC chromatograms for the two feed sources, yeast extract and Primatone, are presented. In general, both feed sources possess the same peaks in their chromatogram. Differences are obtained by comparing the heights of specific peaks; yeast extract has a higher concentration of more hydrophilic compounds than Primatone. In contrast, Primatone shows slightly higher heights for the peaks located on the right side of the chromatogram, representing a higher concentration of hydrophobic compounds [Dong, 2006]. In terms of the relative distribution of peptides, yeast extract contains more hydrophilic than hydrophobic compounds, as the heights of the peaks on the left side of the chromatogram are much higher. Primatone seems more balanced in terms of hydrophilic and hydrophobic compounds. There are still more hydrophilic compounds than hydrophobic ones, but this difference is not as distinct as for yeast extract. From these results it would be expected that both feed sources will behave differently during fractionation by nanofiltration.

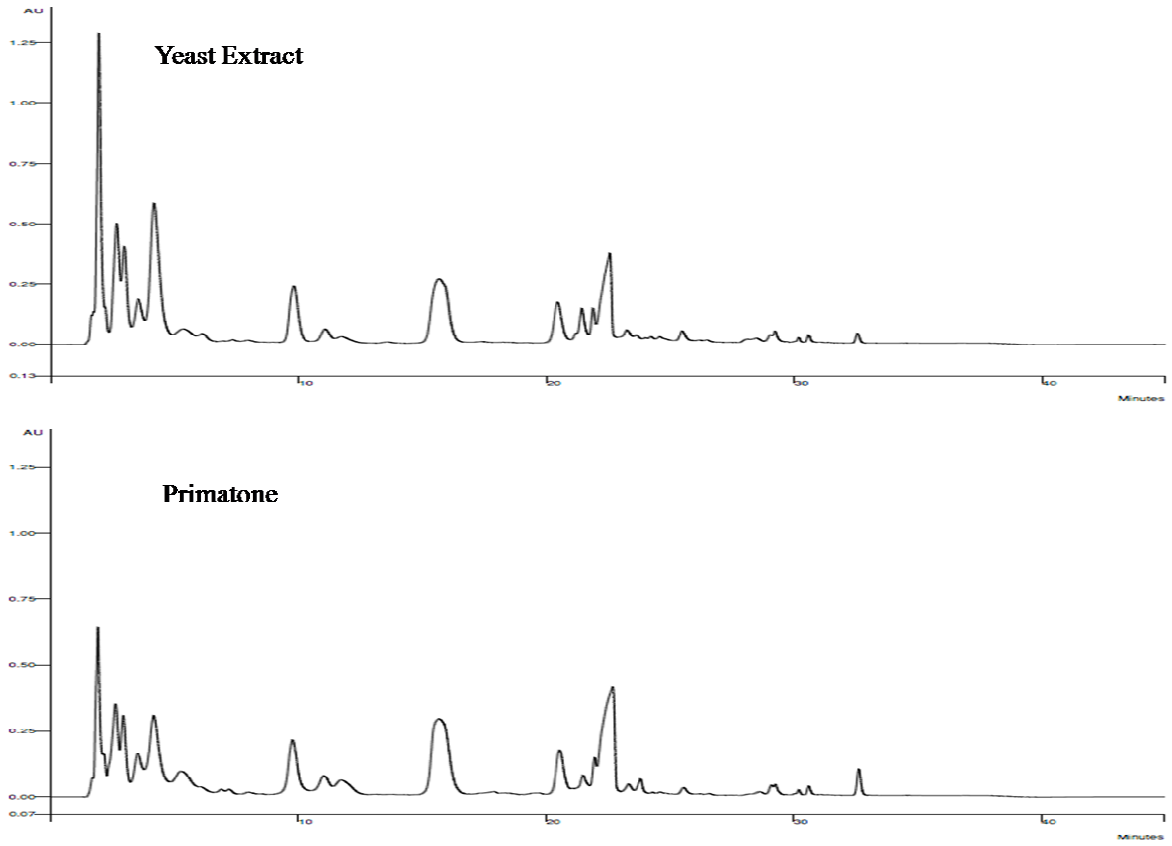

Figure 7.2 Chromatograms of RP-HPLC separation of yeast extract and Primatone

A mass balance for a given filtration was established by adding the height of all the peaks for the retentate and the permeate and subtracting the height of all the peaks for the feed. Some of the peaks in the fractions, especially the ones corresponding to the amino acids observed in the standard, were significantly higher than what was observed in the respective feed. This may be due to the release of amino acids from the peptide breaking-up when exposed to the high pressure of the nanofiltration operation, 2 MPa. As the combined height of some of the peaks in the retentate and the permeate are significantly higher in comparison to the feed sample (20 fold higher in some of the samples) and mostly present in all the samples, it is not assumed to be a procedure artifact or a sensitivity issue. This may also be due to differing molecule elution times overlapping, resulting in higher responses.

7.3.2 PCA analysis

The influence of the different nanofiltration conditions on the amino acids / peptide fractionation, was obtained by principal component analysis. Ordinations were created with all the nanofiltration samples described previously in chapter 6 for two different membranes, two different pH, with or without NaCl addition and two feed sources, yeast extract and Primatone. The RP-HPLC chromatograms profiles of yeast extract and primatone, the retentate and the permeate of the ultrafiltration pretreatment step and all the retentates and the permeates from the nanofiltration experiments were considered. The height of each peak was compared with all the other peaks for a given sample. The variables consisted of 39 peaks with different retention times (Table 7.1) identified acording to their signal strength (7.2.4). Not all peaks were present in all samples.

All data (peak heights) was first transformed by the natural logarithm according to Weber and Legge (2009). Covariances which measures the linear dependence between two variables (in this case the peaks with different retention time) was estimated [Legendre and Legendre, 1998]. Samples closer to each other in the PCA ordinations correspond to fractions with similar peak distribution while fractions with dissimilar peak distribution will be located in different areas of the PCA ordinations. Therefore fractions with similar peak distribution will essentially form groups on the different PCA ordination planes.

As a first comparison a principal component analysis for all the fractions obtained with both feed sources was performed and an ordination created as shown in Figure 7.3. The upper group shown in Figure 7.3 contains all the ultrafiltration fractions of yeast extract and Primatone, creating a group distinct from the nanofiltration fractions. The nanofiltration fractions of yeast extract and the HL membrane were mostly grouped on the left indicating similar characteristics. The nanofiltration fractions of Primatone and the HL membrane, yeast extract and the G-10 membrane, and Primatone and the G-10 membrane, did not show significant grouping.

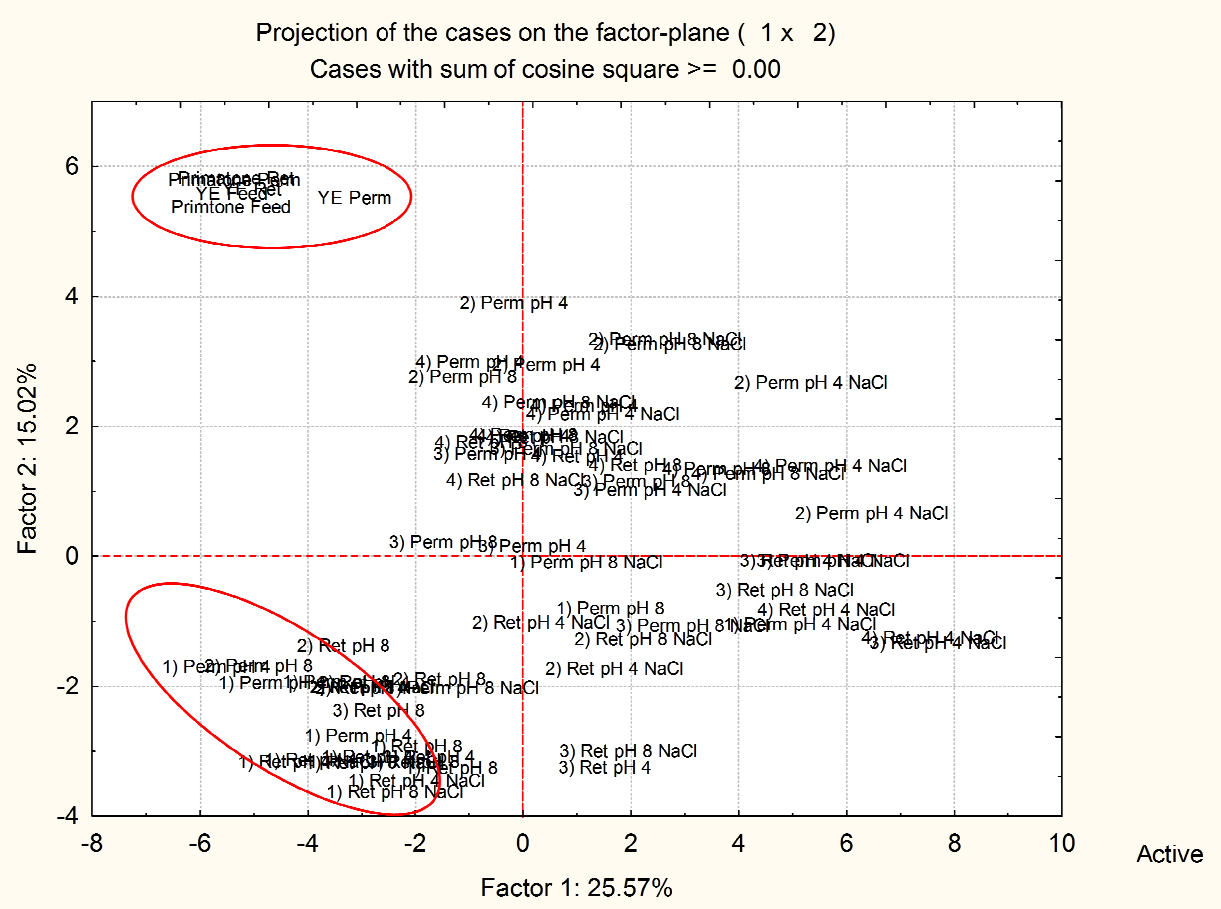

Figure 7.3 PCA ordination of the entire logarithmic transformed data set

Legend: Feed source: YE (yeast extract), P (Primatone); P Permeate, R Retentate; 8 (pH 8) 4 (pH 4); S (NaCl addition); 1 (yeast extract and HL membrane), 2(Primatone and HL membrane, 3 (yeast extract and G-10 membrane), 4 (Primatone and G-10 membrane).

Further PCA analysis was obtained for the data divided in two groups according to the feed source, yeast extract and Primatone. The PCA plot, presented in Figure 7.4 for yeast extract, shows some form of grouping with the retentate fractions for both membranes grouped within the bottom half of the ordination and most of the permeate fractions located in the upper half of the ordination. These results suggest that the peptide profiles for yeast extract were dependant on the separation process with different RP-HPLC profiles for the permeate and the retentate fractions independent of the membrane type.

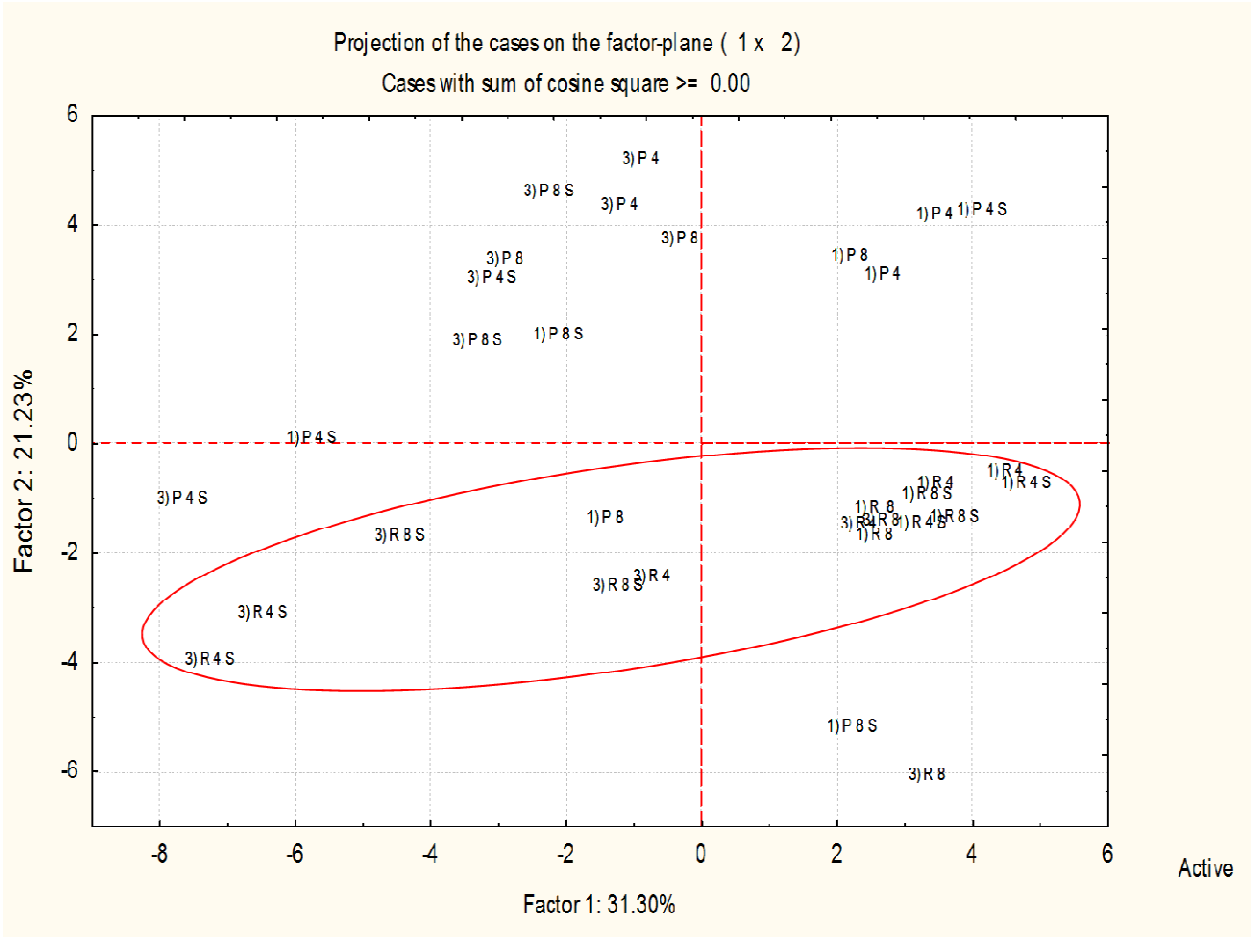

Figure 7.4 PCA ordination of yeast extract and the HL and the G-10 membrane.

Legend: P Permeate, R Retentate; 8 (pH 8) 4 (pH 4); S (NaCl addition); 1 (yeast extract and HL membrane), 3 (yeast extract and G-10 membrane),

The PCA ordination of the Primatone samples fractionated by the HL and G-10 membrane, presented in Figure 7.5, also shows some form of grouping for most of the retentate fractions located in the bottom half of the ordination. Another group with most of the permeate fractions for the G-10 membranes is located in the top half of the ordination. Most other permeate fractions are distributed across the top half of the plot. This suggest that the permeate fractions obtained by the filtration with the G-10 membrane are more similar to each other than the ones obtained with the HL membrane.

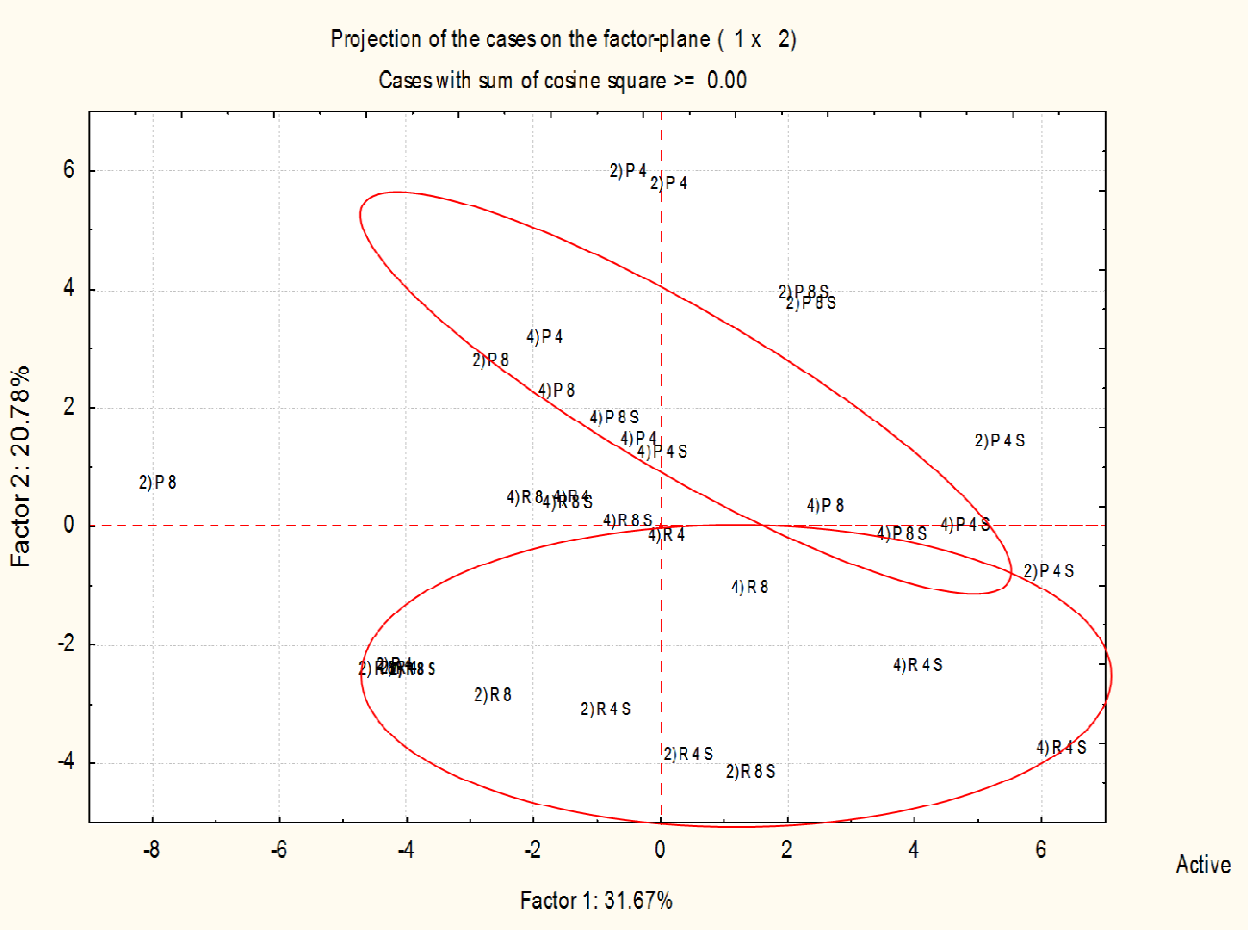

Figure 7.5 PCA ordination of Primatone and the HL and the G-10 membrane.

Legend: P Permeate, R Retentate; 8 (pH 8) 4 (pH 4); S (NaCl addition); 2 (Primatone and HL membrane, 4 (Primatone and G-10 membrane).

The influence of pH or NaCl addition on the RP-HPLC profiles could not be visualzed with the previous plots. Therefore the data sets were further divided. The first set of data, yeast extract separated by the HL membrane, presented in Figure 7.6, shows a tight grouping of all retentate fractions suggesting that the retentate fractions are different from the permeate fractions. Any influence of the pH or NaCl addition could be seen. Moreover the replicates of the 4 different conditions did not aggregate together on the ordination plane which suggests problems with reproducibility. The reason of these problems should be identified in future work and could may be due to separation differences during the filtration, problems in the derivatization procedure, or a problem in the separation by chromatography.

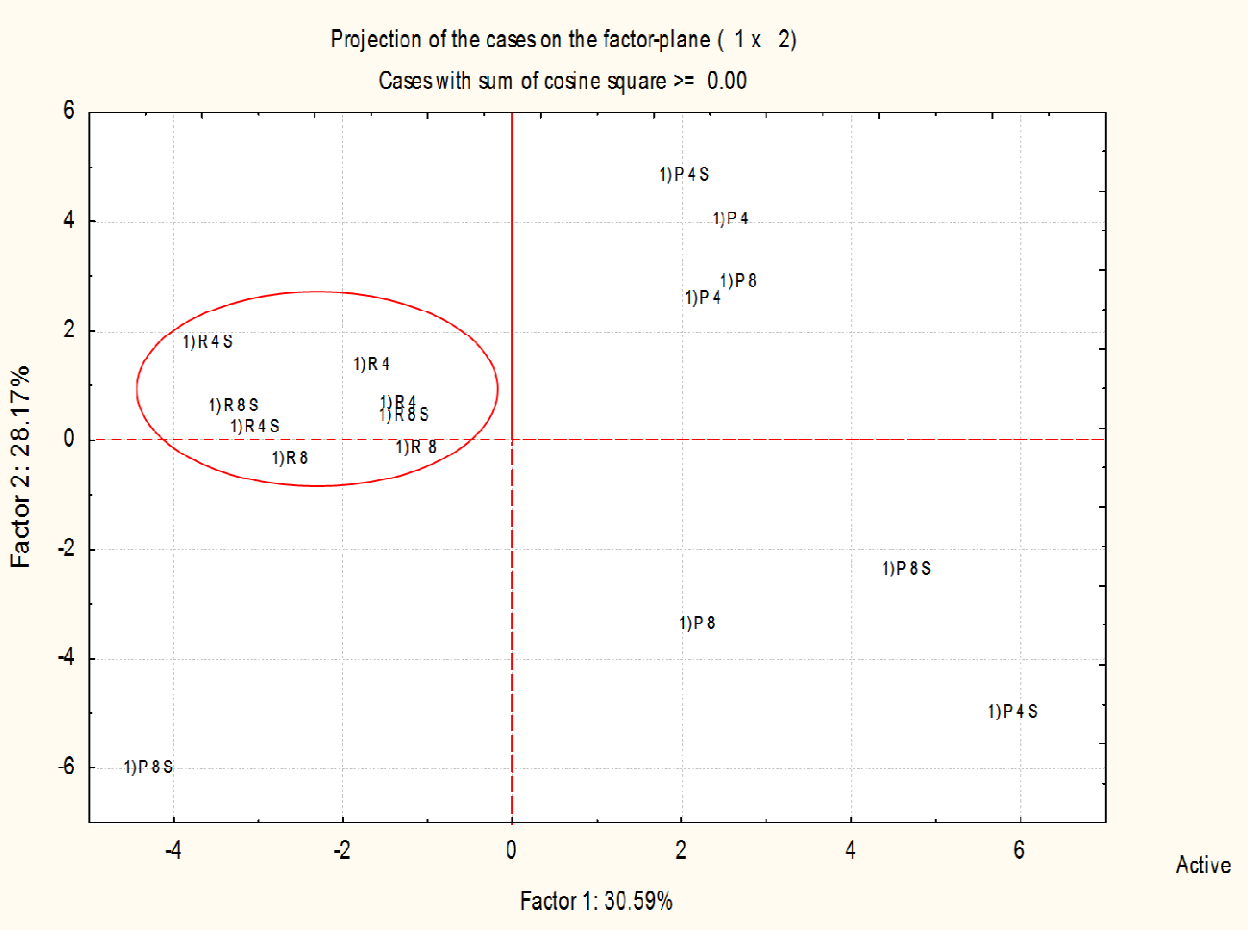

Figure 7.6 PCA ordination of the data foryeast extract and the HL membrane.

Legend: P Permeate, R Retentate; 8 (pH 8) 4 (pH 4); S (NaCl addition); 1 (yeast extract and HL membrane),

The PCA plot for Primatone filtered with the HL membrane, presented in Figure 7.7, shows a group for the retentate fractions. The four permeate fractions, with NaCl addition are grouped together. This suggest that NaCl addition has an influence on the RP-HPLC profile and the corresponding peptide separation. Replicates here were found to be more similar to each other than for yeast extract and the HL membrane.

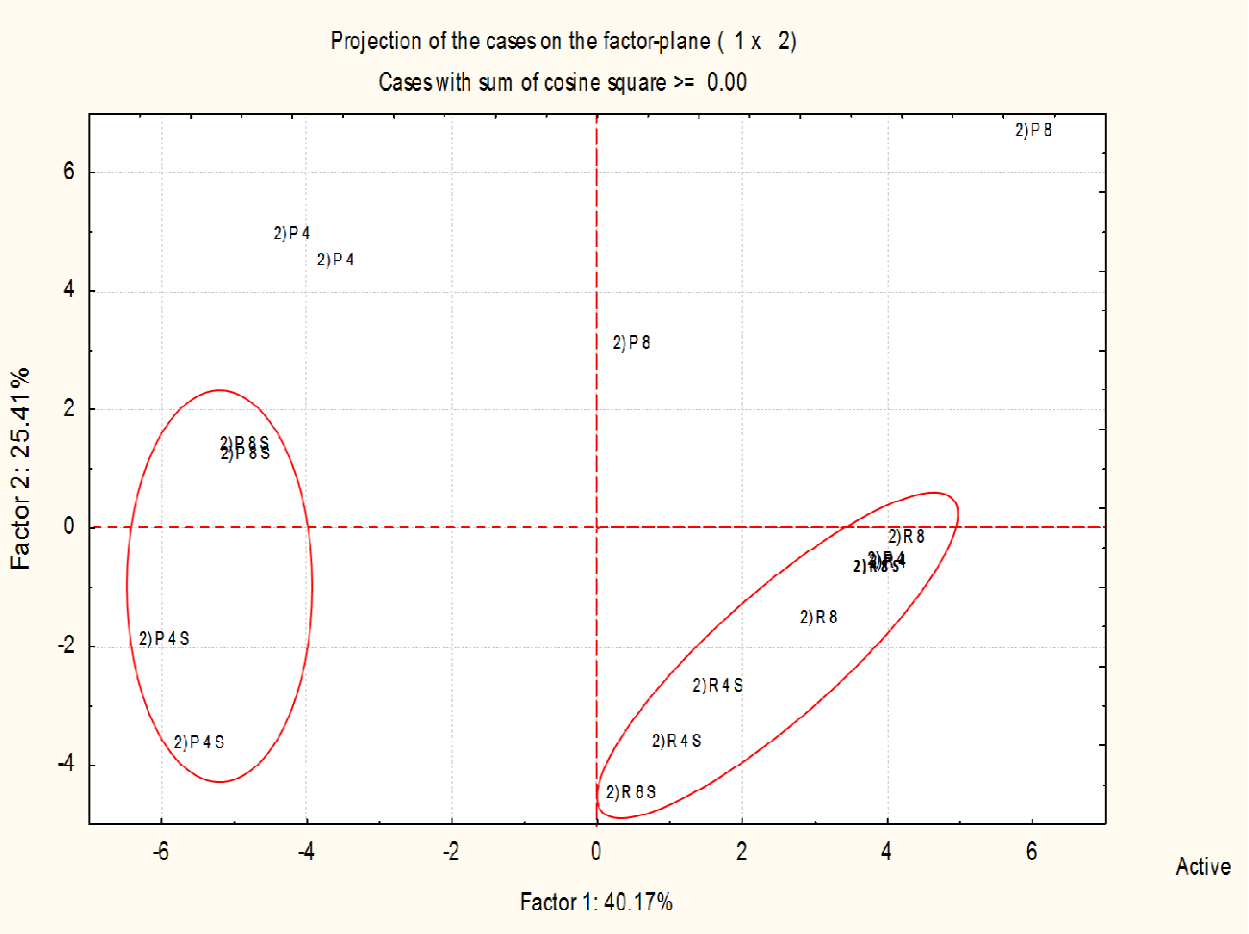

Figure 7.7 PCA plot of Primatone and the HL membrane

Legend: P Permeate, R Retentate; 8 (pH 8) 4 (pH 4); S (NaCl addition); 2 (Primatone and HL membrane, 3

The PCA ordination for yeast extract separated through the G-10 membrane, presented in Figure 7.8, indicates differences between the retentate fractions which could not be grouped together. Most of the permeate fractions grouped on the right side of the plot, include permeate fractions performed at pH 8. For yeast extract and the G-10 membrane, pH shows an effect on the selectivity of the peptide transmission.

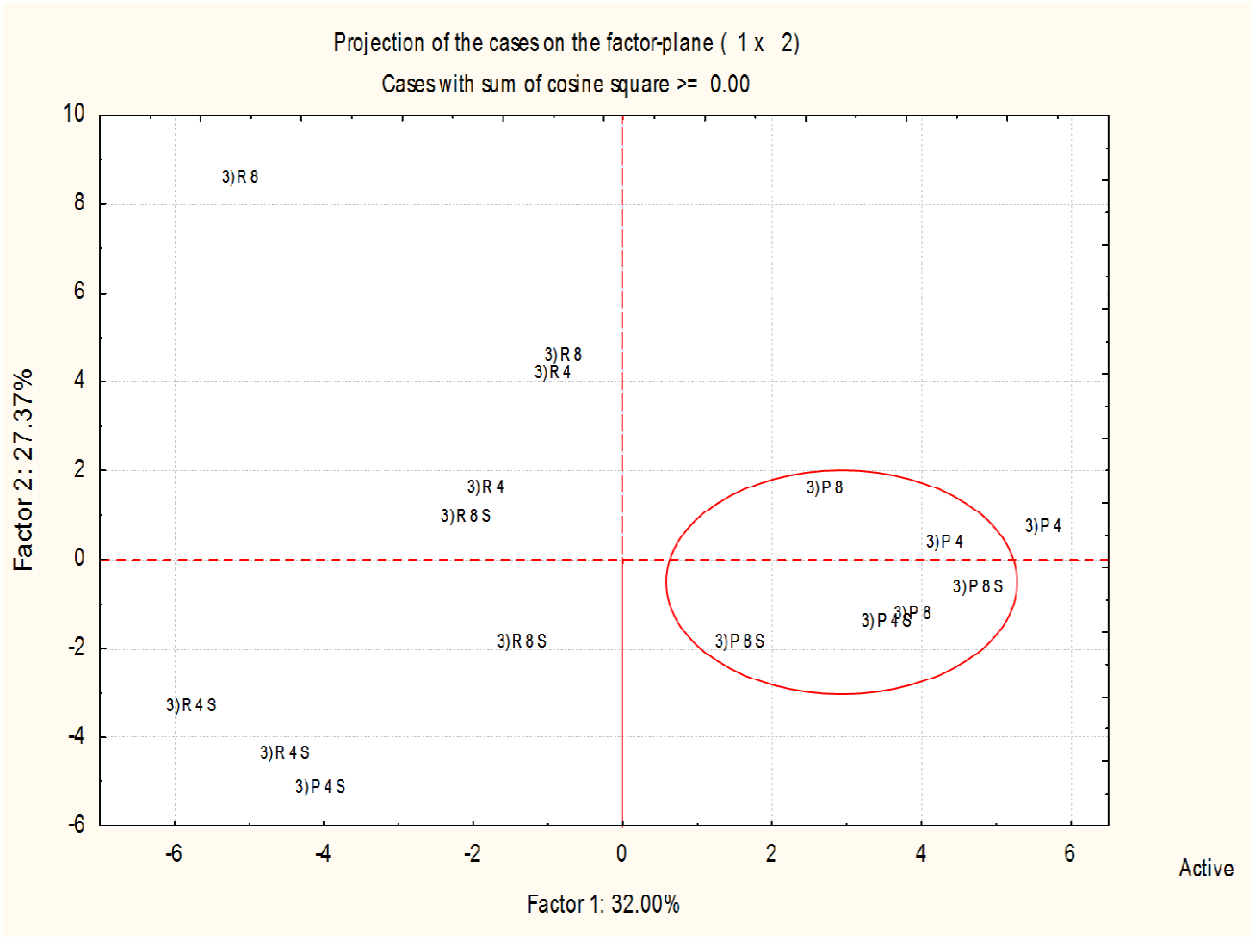

Figure 7.8 PCA plot of the data for yeast extract and the G-10 membrane.

Legend: P Permeate, R Retentate; 8 (pH 8) 4 (pH 4); S (NaCl addition); 3 (yeast extract and G-10 membrane)

The PCA ordination for Primatone separated with the G-10 membrane, presented in Figure 7.9, does not reveal any significant grouping. The retentate fractions are located mainly in the lower part of the plot and the permeate fractions mainly in the upper part. Also the replicates did not aggregate with each other on the plot. It should also be noted that a significant number of peaks (7 out of 39) were not present in all fractions.

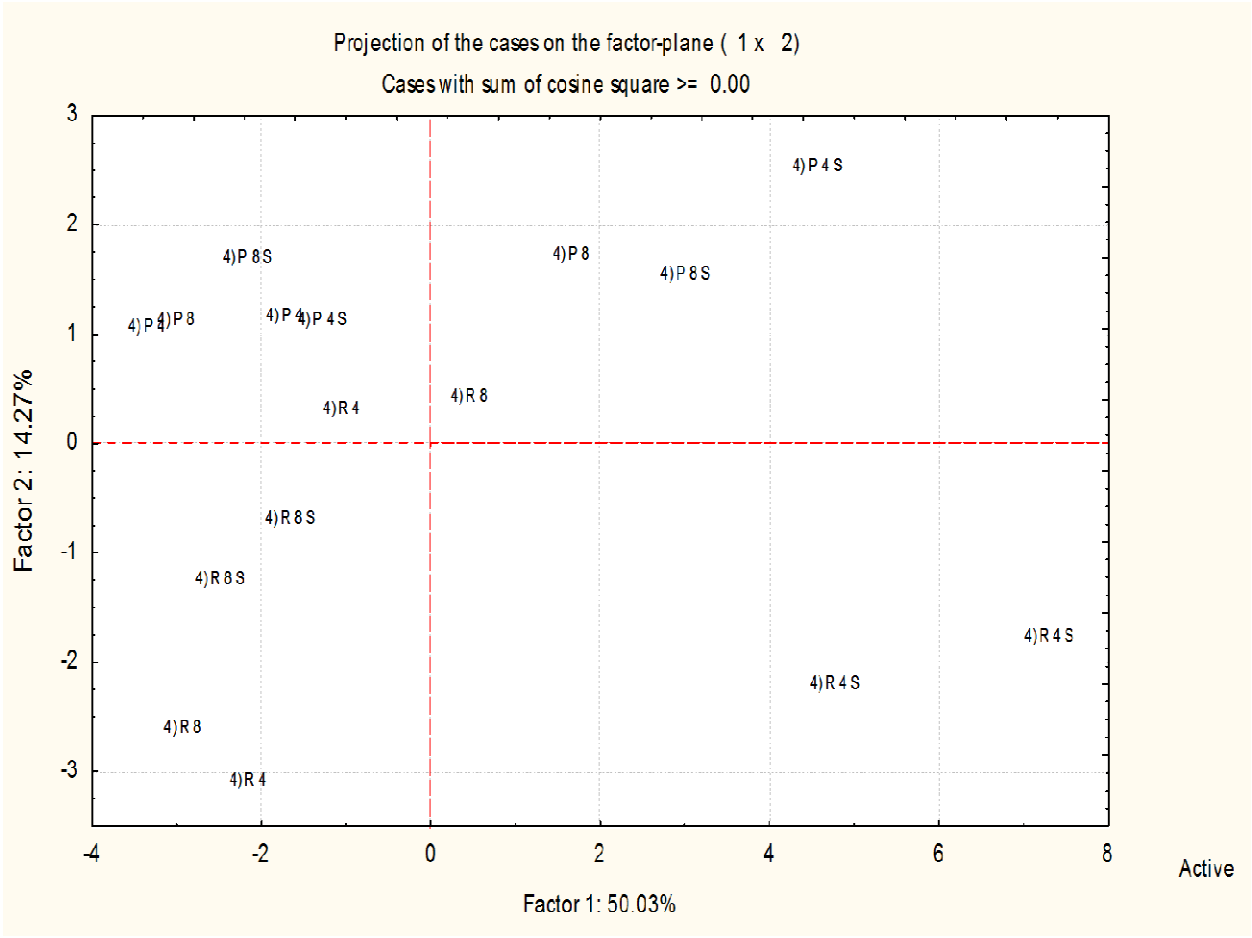

Figure 7.9 PCA plot of the data for Primatone and the G-10 membrane.

Legend: P Permeate, R Retentate; 8 (pH 8) 4 (pH 4); S (NaCl addition); 4 (Primatone and G-10 membrane).

For the ultrafiltration fractions a separate PCA plot was established and presented in Figure 7.10. The primatone fractions are grouped in the lower part of the right side of the ordination whereas the yeast extract fractions are more distributed accross the plot. This suggests a difference in the peptide profile for Primatone and yeast extract. Moreover, it is visible that the yeast extract samples are further apart from each other which suggests a similar peptide profile for Primatone.

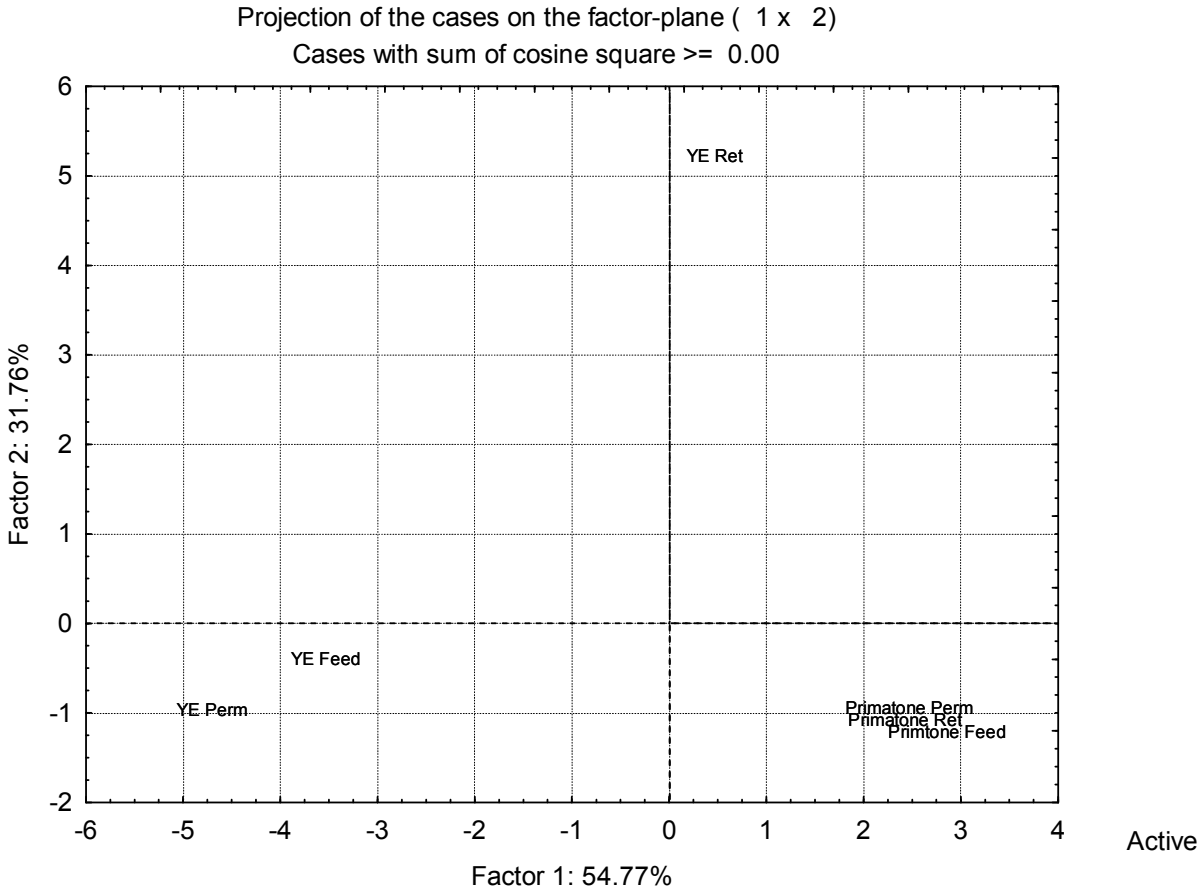

Figure 7.10 PCA plot of the data for the ultrafiltration fractions.

Legend: YE (yeast extract), Prim (Primatone), Perm (Permeate), Ret (Retentate)

7.4 Conclusions

RP-HPLC was used to investigate the peptide profile present in different fractions of yeast extract and Primatone obtained with ultra and nanofiltration. The effect of the different nanofiltration conditions (membrane type, feed source, pH, NaCl addition) on the amino acids / peptide fractionation was further investigated by principal component analysis.

The peptide distribution according to relative hydrophobicity for yeast extract and Primatone was obtained by RP-HPLC. Yeast extract contains more hydrophilic compounds than hydrophobic ones. Primatone on the other hand is more balanced with its composition.

In establishing a mass balance with the peak height for the nanofiltration permeate and retentate fractions according to feed composition, amino acids / peptides were produced. This may be due to the break-up of peptides into smaller peptides and single amino acids when subjected to the high pressure of the nanofiltration operation. The peak intensity was more pronounced for the peaks corresponding to the individual amino acids, which suggests further break-up of smaller peptides into amino acids. However as more than 20 peaks could be identified in each chromatogram, it is assumed that peptides were also present. No study has been published investigating the effect of high pressure operation on peptide stability.

The comparison of the heights for the major peaks (39 peaks maximum) detected by RP-HPLC for yeast extract and Primatone and their corresponding ultrafiltration and nanofiltration fractions by principal component analysis revealed significant differences on the characteristics of the peptides. The peptide characteristics of the fractions produced by ultrafiltration are distinct from the nanofiltration fractions. This shows that the nanofiltration has an effect of the amino acid and peptide distributions and selectively transmits some components in comparison to ultrafiltration. Information on the influence of pH and NaCl addition was obtained by looking at four sub-sets of the full factorial design. For both types of feed separated with the HL membrane, the retentate fractions could be grouped together suggesting similarity in the peptide hydrophobicity characteristics. For Primatone separated with the HL membrane, the permeate fractions with NaCl addition constituted a group, suggesting that salt addition had an influence on the transmission of peptides with similar

hydrophobicity pattern. Furthermore, for yeast extract separated with the G-10 membrane, the permeate fractions at pH 8 were grouped together. This suggest that the pH had an influence of the separation of peptides with similar hydrophobicity characteristics for this membrane and feed type.

According to the PCA ordinations, each membrane separates peptides and amino acids differently and this is related to the feed source, pH and NaCl addition. The different feed sources likely have different charges on the different molecules present in the mixture, therefore it was not unexpected to see a difference in the peptide distributions. As it was shown in the analysis of the feed of yeast extract and Primatone, the composition of peptides / amino acids is different in both sources. Primatone and yeast extract are both from hydrolysed sources however the former being derived from an animal source whereas the latter is derived from yeast cells.

Although the molecular weight of the peptides could not be determined because of the lack of a mass spectrometer it was possible to see a separation of amino acids and peptides by RP-HPLC without a MS. The advantage of having a MS would be that each obtained molecular weight of a peptide could be attributed to a sequence of amino acids, compared to an available data base.

Future work should focus on the isolation and characterization of the individual peptides contained in the different fractions. Each individual peptide and their combination could be investigated for their effect on cell growth and productivity. The isolation, characterization, and assessment for all the different peptides and their combination would be labor intensive. But this work would help in the development of a relatively cheap, chemically defined serum-free media. If peptides could be isolated and characterized, the addition of these peptides would be accepted as chemically defined by the U.S. Food and Drug Administration (FDA).

Chapter 8: Investigation of the Nanofiltration Fractions as a Viable Nutrient Media Additive in Chinese Hamster Ovary Cells

Overview

The fractions obtained by nanofiltration for yeast extract and Primatone were investigated for their enhancing effect on CHO cell growth and β−interferon productivity. Previous studies have shown the beneficial effects of the addition of protein hydrolysates to improve cell growth and therapeutic proteins productivity in some cell lines grown in serum-free media. In the current study, the retentate fraction for yeast extract separated with the G-10 membrane at pH 8 showed the highest overall enhancing effect (cell density) tested in CHO cell culture and was even higher than a positive control containing 0.2% Primatone. Primatone is assumed to increase the cell growth significantly and was used as a positive control reference. Although a low cell density was observed in the retentate for yeast extract separated with the HL membrane at pH 4 and with NaCl addition, the highest specific productivity was achieved, suggesting that the productivity was not related to the cell density in this situation. The detailed analysis of the reproducibility and stability in maintaining the growth of CHO cell culture for each fraction is required to conclude the optimal condition for a nanofiltration fractionation. The preliminary results indicate that the fractionation of protein hydrolysates by nanofiltration is an interesting tool in enriching protein hydrolysates for their biological activity.

8.1 Introduction

In this study, a CHO cell line producing β−interferon as a therapeutic protein was chosen for the investigation of yeast extract and Primatone fractions obtained from nanofiltration as supplements in serum-free media. Interferons belong to the family of cytokines which are produced naturally in various cells of the immune system in response to biological or chemical influences such as viruses, parasites, or tumor cells. Different kinds of therapeutic interferon pharmaceuticals are on the market to treat diseases. They have antiviral, antiproliferative, and immunomodulatory properties [Arduini et al., 2004]. β−interferon contains 166 amino acids and is used in the treatment of multiple sclerosis [Meager and Das, 2005].

Although the expression of therapeutic proteins in mammalian cells has advantages such as proper folding of the protein and post-translational modification, it is difficult to achieve a stable cell line which expresses the introduced gene in a high quantity [Kim et al., 2005]. The complex media requirement of mammalian cells is also often a challenge. As serum should be avoided as a media additive according to the FDA, a serum-free media should be developed. Avoiding serum, however, often results in lower cell densities and production rates. Protein hydrolysates are attractive as supplements for serum-free media and have been shown to stimulate the growth of certain mammalian cell lines. The addition of yeastolate, whey gluten hydrolysate and soy protein hydrolysate have shown to enhance the growth and productivity of certain cell lines [Shen et al., 2007, Farges-Hadani et al., 2006, Sung et al., 2004 , Franek et al., 2000]. Some of the work presented by these authors also investigated the difference between crude hydrolysate and fractionated hydrolysate by SEC or ultrafiltration. Low molecular fractions were more stimulating than high molecular weight fractions but in comparison to the crude hydrolysate still showed a lower enhancing effect [Mendonça et al., 2007].

The fractionation of peptide mixtures by nanofiltration and the subsequent investigation of the fractions in mammalian cells has not been investigated yet. As yeast extract and Primatone are both considered to enhance cell growth of mammalian cells, these two feed sources were investigated for fractionation by nanofiltration and investigate for their effect in CHO cell culture.

8.2 Experimental

All experiments were performed at the University of Manitoba by Dr. Mike Butler and Vincent Jung.

8.2.1 Cell Cultures

The CHO cell line producing β–interferon was seeded at 10^5 cells/mL in a 25 cm^2 T-flask with 4 mL of medium. The basal medium was Biogro-CHO (Biological Industries, Kibbutz Beit Haemek, Israel) and supplemented with 0.025 %w/v of the specific fraction obtained from the nanofiltration. The freeze-dried nanofiltration fractions were dissolved and added as a supplement directly to the cell culture. Each culture condition was performed in duplicate. The cells were grown at 37°C in an incubator with 5% CO_2 over a 4 day period. Each day, the cell concentration was determined by trypan blue coloration (1 part sample + 1 part 0.2 %wt trypan blue) and counting the viable unstained cells in a haemocytometer. At day 4, the cell concentration was determined by trypan blue coloration and seeded again at 10^5 cells/mL in a new 25 cm^2 T-flask with 4 mL of fresh medium containing the same hydrolysate fraction. The last procedure was performed once again until three sub-culturing steps were obtained. Three sub-culturing steps (passages) could not be performed with all samples (n = 6). The number of passages that was performed for each of the tested fraction is shown in Table 8.1.

As a negative control the same procedure was performed without the addition of a specific fraction (cells cultured in basal media only). As a positive control, the addition of 0.025% w/v crude yeast extract or 0.2% crude Primatone was added to the basal media.

Table 8.1 Number of passages that were performed for each cell culture condition

Sample	Number of Passages
Hydrolysatefree (negative control)	3
0.2% primatone (positive control)	3
0.025% Yeast Extract (positive control)	3
UF 10 kDa Permeate (NF feed)	3
Yeast Extract Retentate pH 4 HL membrane	3
Yeast Extract Retentate pH 8 HL membrane	3
Yeast Extract Retentate pH 4 NaCl HL membrane	2
Yeast Extract Retentate pH 8 NaCl HL membrane	3
Yeast Extract Permeate pH 4 HL membrane	3
Yeast Extract Permeate pH 8 HL membrane	2
Yeast Extract Permeate pH 4 NaCl HL membrane	2
Yeast Extract Permeate pH 8 NaCl HL membrane	2
Primatone Retentate pH 4 HL membrane	2
Primatone Retentate pH 8 HL membrane	2
Primatone Permeate pH 4 HL membrane	1
Primatone Permeate pH 8 HL membrane	2
Yeast Extract Retentate pH 4 G-10 membrane	2
Yeast Extract Retentate pH 8 G-10 membrane	1
Yeast Extract Permeate pH 4 G-10 membrane	1
Yeast Extract Permeate pH 8 G-10 membrane	2
Primatone Retentate pH 4 G-10 membrane	0
Primatone Retentate pH 8 G-10 membrane	2
Primatone Permeate pH 4 G-10 membrane	2
Primatone Permeate pH 8 G-10 membrane	2

8.2.2 ELISA

The productivity of β−interferon was measured in the supernatant of the cell suspension after a centrifugation at day 4 by an enzyme linked immunosorbent assay (ELISA). Each sample was measured in duplicates at 8 different dilutions (2 / 4 / 8 / 16 / 32 / 64 / 128x diluted) in a microtiterplate according to the procedure described in Tharmalingam et al. (2008). A specific productivity was calculated according to equation 8.1.

$$P_S = \frac{P}{CD*V*t} \quad (8.1)$$

Where P_S = Specific productivity [units β-interferon/(cells*day)]

P = Productivity [units β-interferon]

CD = Cell density [cells/mL]

V = Volume [mL]

t = Time to reach the cell density CD [day]

Only the samples listed in Table 8.2 were anlyzed for β–interferon concentration present in the media.

Table 8.2 Samples for the productivity measurement of β–interferon by ELISA

Sample
Fraction free (negative control)
0.2% Primatone (positive control)
0.025% Yeast Extract (positive control)
0.05% Yeast Extract (positive control)
UF 10 kDa Permeate (NF feed)
Yeast Extract pH 4 Retentate HL membrane
Yeast Extract pH 8 Retentate HL membrane
Yeast Extract pH 4 NaCl R HL membrane
Yeast Extract pH 8 NaCl R HL membrane
Yeast Extract pH 8 NaCl Permeate HL membrane

8.3 Results and Discussion

8.3.1 Growth curves

To analyse the effect of the nanofiltration fractions on the growth behaviour of CHO cell culture, the retentate and permeate fractions of the nanofiltration experiments were added in addition to the basal media and the growth was monitored during a 4 day period.

The sub-culturing or passaging of cells results in the stability of the cells, and the ability of growing for a longer time in a media supplemented with a specific fraction can be monitored as well. Not all samples were passaged 3 times; therefore the reproducibility of the samples with a lower passage number is not assured. Also some of the samples with 3 passages had a very high standard deviation (not shown on the graphs). Thus, only the average of the available replications of the sub-cultures for each sample is presented in Figures 8.1 – 8.7. A higher standard deviation is tolerated in living organisms such as cells as each cell is considered to be a distinct organism.

The samples containing 150mM NaCl were not desalted before freeze drying. Therefore the salt was present in a high concentration after the freeze drying. As the fraction was dissolved in a solution based on weigth to volume ratio, the peptide content of these fractions is therefore lower than for the fractions with no NaCl addition. It would be expected that the fractions with NaCl addition will show a lower effect in the growth enhancing as the peptide concentration is lower and the high concentration of salt could even inhibit growth. Only the yeast extract fractions filtered by the HL membrane that contained salt were analyzed.

The effect of the membrane type on cell growth and the yeast extract filtered with the HL membrane and the G-10 membranes is presented in Figure 8.1. In general the fractions filtered with the G-10 membrane show more pronounced cell growth enhancement. The retentate fraction at pH 8 resulted in the highest cell density, $4.6*10^{-6}$ cells/mL after 4 days, which is twice the cell density of the positive control containing crude yeast extract. Although this sample had only one passage, the growth enhancing effect was significant. A further

investigation with more passages would be necessary to see the stability of this substantially increased cell growth rate.

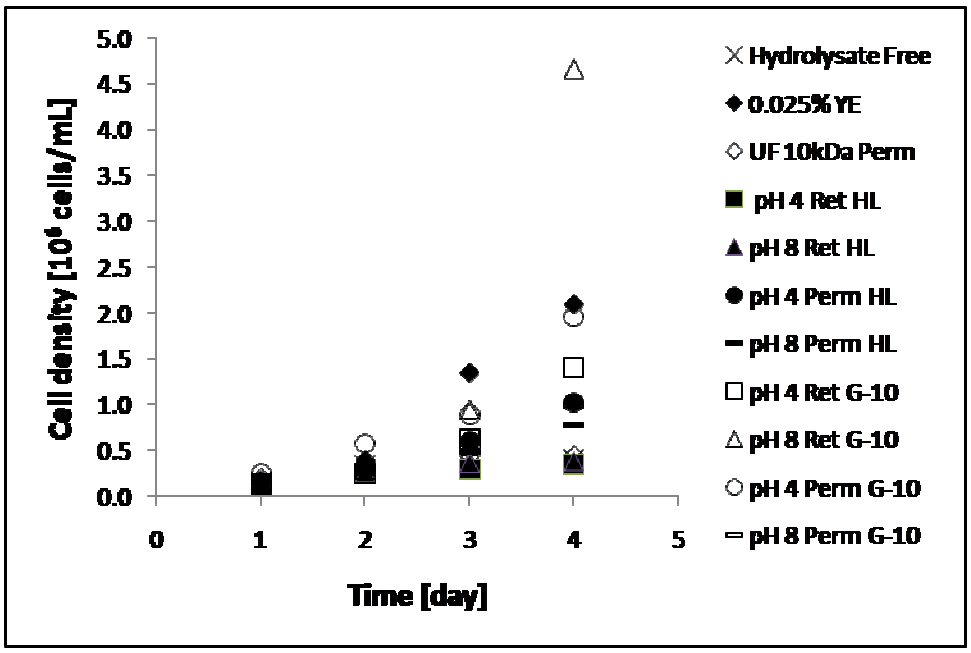

Figure 8.1 Cell densities of CHO cells during a 4 day period and grown with different fractions obtained by nanofiltration (Yeast extract with the HL and G-10 membrane)

In contrast to yeast extract, Primatone filtered with the HL shows a more significant growth enhancing effect than for the G-10 membrane (Figure 8.2). The hydrolysate free sample showed the lowest cell density of all the samples. Furthermore, the positive control showed the highest cell growth rate, but was also added in a 8 times higher concentration than the fractions.

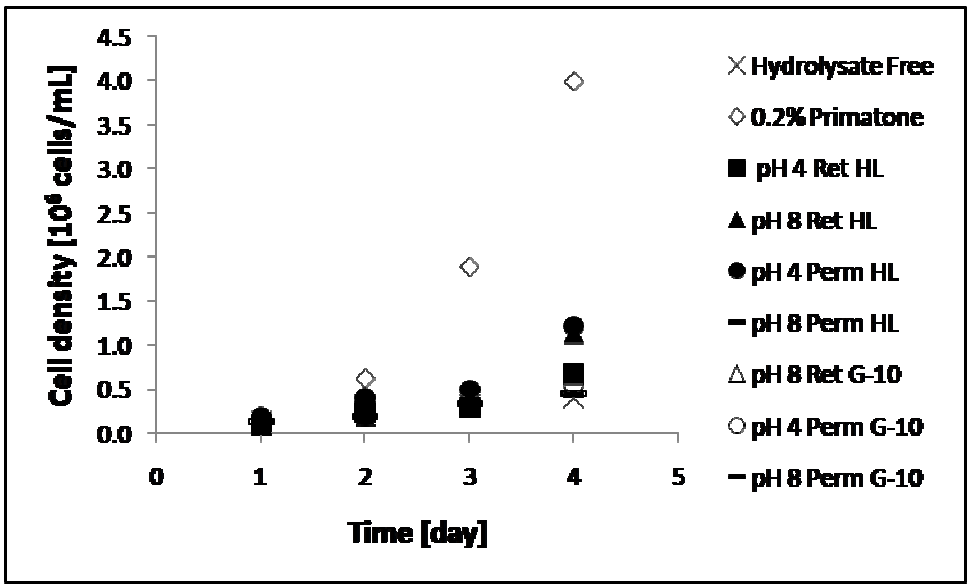

Figure 8.2 Cell densities of CHO cells during a 4 day period and grown with different fractions obtained by nanofiltration (Primatone with the HL and the G-10 membrane)

To investigate the effect on the cell growth rate for the retentate and permeate fractions obtained at different pH, the data was grouped according to feed source and membrane type.

Figure 8.3 presents yeast extract filtered with the HL membrane. As expected, the fractions containing salt had a lower or no effect in stimulating the cell growth. These four samples show a similar or lower effect than the hydrolysate free culture. On the other hand, the permeate fractions at pH 4 and pH 8 showed a higher cell density but lower than the positive control with crude yeast extract. The permeate fraction of the ultrafiltration fractionation resulted in a cell density similar to the one observed with the hydrolysate free culture.

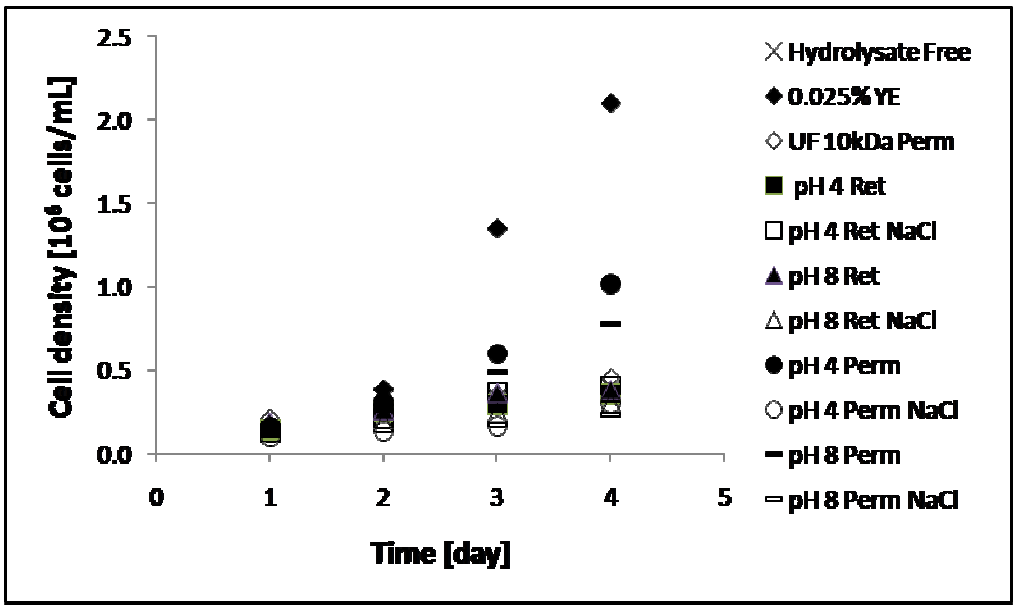

Figure 8.3 Cell densities of CHO cells during a 4 day period and grown with different fractions obtained by nanofiltration (yeast extract and the HL membrane)

As the presence of salt seem to have a limited or negative effect on cell growth, the other fractions containing salts were not investigated.

The Primatone retentate fraction at pH 8 and permeate fraction at pH 4 obtained with the HL membrane (Figure 8.4) displayed the best stimulating growth enhancing effect. The cell density obtained after a 4 day period was significantly higher than the hydrolysate free culture samples according to the t-test ($\alpha = 0.05$, one tail).

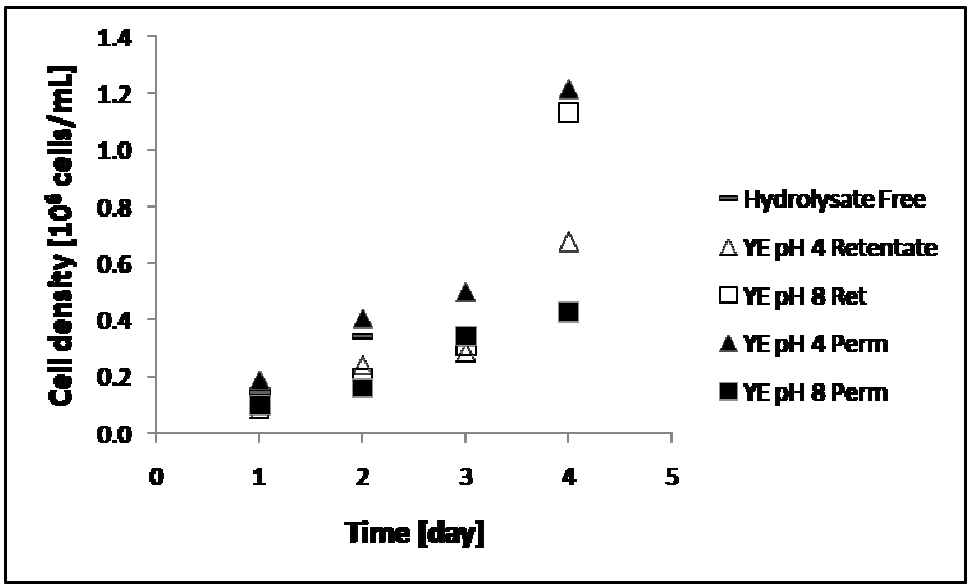

Figure 8.4 Cell densities of CHO cells during a 4 day period and grown with different fractions obtained by nanofiltration (Primatone and the HL membrane)

Figure 8.5 summarizes the cell densities for the yeast extract separated with the G-10 membrane. The retentate at pH 8 shows a significantly higher cell density than all other samples. All fractions resulted in a higher cell density than the permeate fraction of the 10kDa ultrafiltration (Nanofiltration feed) which shows that the nanofiltration has some positive effect on the growth enhancing effect of the fractions.

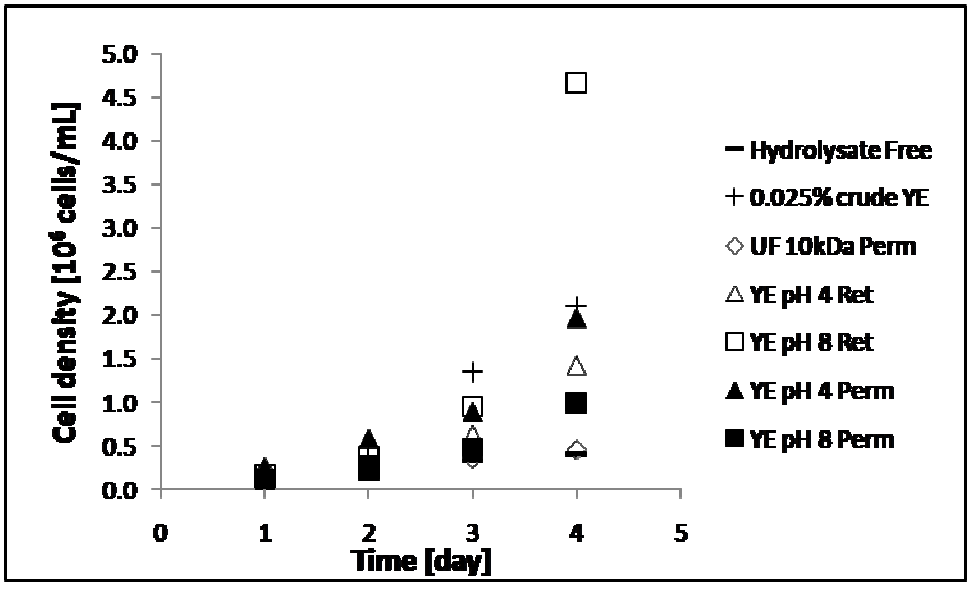

Figure 8.5 Cell densities of CHO cells during a 4 day period and grown with different fractions obtained by nanofiltration (Yeast extract and the G-10 membrane)

Considering the large standard deviations (data not shown), the Primatone fractions obtained with the G-10 membrane (Figure 8.6) showed similar cell densities as the hydrolysate free sample. Therefore, it could be concluded that the separation of Primatone by the G-10 membrane did not result in any growth stimulating effect.

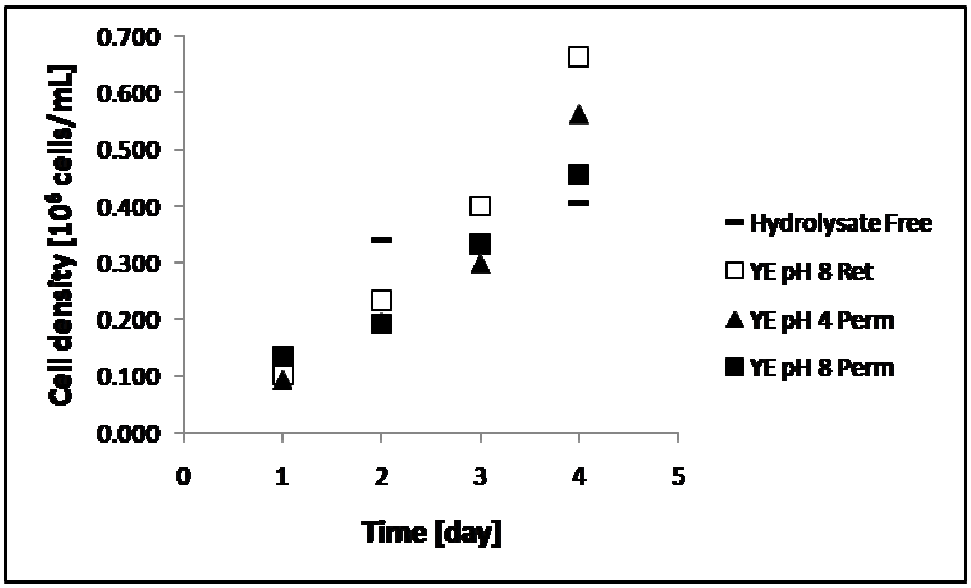

Figure 8.6 Cell densities of CHO cells during a 4 day period grown with different fractions obtained by nanofiltration (Primatone and the G-10 membrane)

The addition of different protein hydrolysates such as yeastolate, whey gluten hydrolysate and soy protein hydrolysate to serum-free media have proven stimulating effects in the growth and productivity of certain cell lines [Farges-Hadani et al., 2006, Sung et al., 2004 , Franek et al., 2000]. Moreover, the low molecular weight fractions obtained by ultrafiltration or SEC fractionation were more stimulating than the high molecular weight fractions as investigated by Mendonça et al. (2007). Therefore, as could be shown in this study by the preliminary results, the fractionation of protein hydrolysates (yeast extract) by nanofiltration is a promising method to separate peptides according to their charge and size.

According to the literature Primatone is assumed to significantly stimulate the cell growth and productivity of certain cell lines and was therefore chosen as a reference [Schläger et al., 1996].

8.3.2 β–Interferon Productivity

A CHO cell line expressing β–interferon was used to determine the productivity in presence of different nanofiltration fractions. The productivity of this cell line was measured according to the concentration of β–interferon present in a sample.

Preliminary results for the fractions obtained with the HL membrane are presented in Figure 8.7. The specific productivity is the highest for the retentate at pH 4 and with the addition of 150mM NaCl. Even if the cell density was low in comparison to other samples (similar to the hydrolysate free sample), the productivity shows a significant increase according to the t-test ($\alpha = 0.05$, one tail). This may be due to a specific peptide or peptide combination present in this fraction. Because of the high salt content remaining after the freeze drying step and the sample preparation according to a prescribed total mass to volume ratio, the peptide concentration is lower than in fractions containing no salt. Therefore the productivity of the cells may have been stimulated by specific peptides present in a low concentration.

The retentate fractions obtained at pH 8 and containing no salt showed a similar (according to the t-test, $\alpha = 0.05$, one tail) productivity as the positive controls and the negative control. The feed of the nanofiltration (Permeate of 10kDa ultrafiltration) also increased the productivity significantly. It appears that the productivity was increased with the addition of both ultrafiltration and nanofiltration fractions.

As only preliminary results are available, the importance of the different conditions for the nanofiltration fractionation is not clear yet. According to the preliminary results presented in this section, the nanofiltration constitutes an interesting method to separate different groups of peptides with different compositions according to the membrane type.

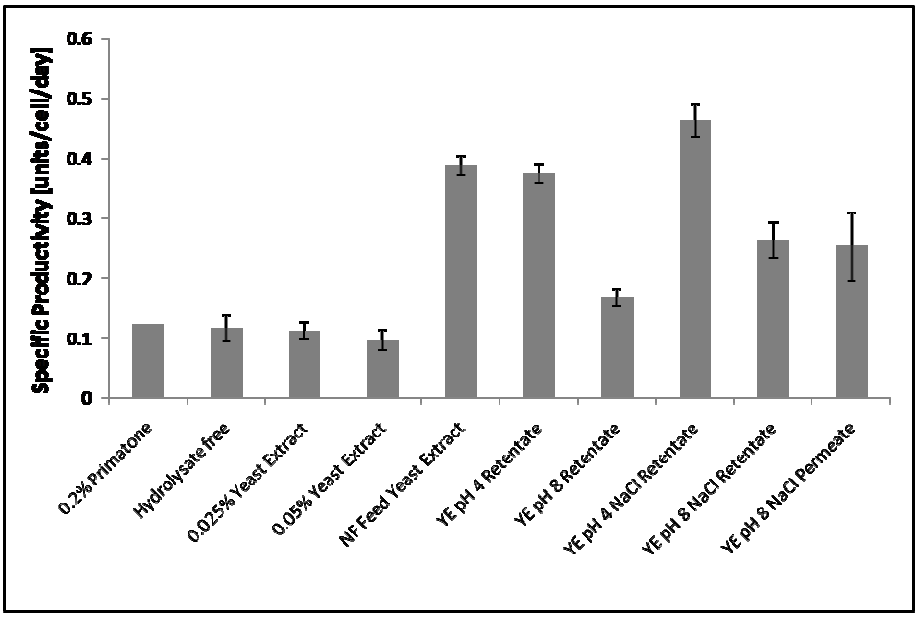

Figure 8.7 Specific β–interferon productivity of CHO cells with the addition of different fractions (n = 2)

8.4 Conclusions

Preliminary results on the investigation of the growth and productivity enhancing effects for different nanofiltration fractions in cell culture were summarized in this chapter.

According to the preliminary results, the retentate fraction of yeast extract separated with the G-10 membrane showed the highest cell density after a 4 day time period. This cell density was higher than the positive control (0.2% Primatone). Primatone is assumed to act as a reference and is considered to enhance significantly cell growth of certain cell lines. Other conditions for the fractionation of yeast extract with the G-10 membrane should be investigated to find an optimal fraction that stimulates the cell growth of CHO cells.

The reproducibility of the cell density for the different sub-cultures was poor and resulted in a high standard deviation. A higher standard deviation between different cell samples is tolerated, as cells are living organisms and are different from each other. But such a large standard deviation limits the analysis of the significant differences due to the presence of a given fraction.

As not all nanofiltration fractions have been tested with cell culture or only 1 passage was performed, one can not identify the optimal nanofiltration conditions for the highest growth enhancing effects. Yeast extract is prefered as an additive to a serum-free medium, as Primatone is of animal source. Therefore, further investigation of a fractionation of yeast extract by the G-10 membrane could result in an optimal enhancing peptide fraction.

The highest specific β-interferon productivity was obtained with the retentate fraction at pH 4 and with the addition of 150mM NaCl. This is surprising considering the presence of a high NaCl concentration in the sample and a lower total peptide concentration when dissolved in a solution based on specified total mass to volume ratio. Further work should include a desalting step before the addition of the fraction to the cells as a high salt concentration could inhibit the growth of the cells completely. The comparison of the same fraction with and without desalting should also be considered. These preliminary results suggest that a high productivity is not always related to the cell density and growth rate.

A further investigation of the productivity of all fractions is also required in order to identify the optimal fractionation conditions that stimulates cell growth and productivity. The separation of yeast extract with the G-10 membrane shows promising results in the cell density, therefore it could be possible to yield a higher productivity as well.

A fraction with a high productivity would be worthwhile to analyze in depth for its peptide content. An analysis by HPLC-MS or MALDI-TOF would identify the peptides present in the mixture and could lead to their separation and purification. The investigation of the addition of single peptides or a combination of different peptides might result in a stimulating effect.

Chapter 9: Overall Conclusions and Recommendations

9.1 Overall Conclusions

The overall objective of this thesis was the investigation of nanofiltration performance on peptide mixtures in order to develop an additive for a serum-free media for CHO cell culture.

The first step in this thesis was the characterization of five nanofiltration membranes in order to choose two of them for the investigation of their fractionation behaviour with peptide mixtures. All of the five membranes tested displayed hydrophilic properties as deduced from their contact angle, and therefore should exhibit low fouling properties. The G-10 and the HL membrane (both thin film composite material) were chosen according to their very different reported MWCO (2500Da and 300-500Da respectively), zeta potential (-72mV and -5mV at pH 8 respectively) fouling potential (0.27 and 0.17 respectively) and transmission of total solids (30% and 8% respectively).

The pretreatment step for the nanofiltration operation was achieved with a 10kDa UF membrane at a constant TMP of 65kPa and at a feed concentration of 5wt% as was identified as appropriate conditions. Yeast extract showed significantly more fouling than Primatone, which was related to a difference in composition of the two feed sources, however both sources resulted in a recovery of around 70% of the initial total peptide concentration and around 65% of total solids in the permeate fraction.

A 2^4 factorial design was adopted for the nanofiltration fractionation of yeast extract and Primatone and the investigation of the effect of membrane type, feed composition, pH and NaCl addition. The nanofiltration performance was measured by the total peptide transmission, organic content transmission, inorganic content transmission, and antioxidant capacity transmission. The HL membrane displayed a significant lower total peptide transmission (around 10%) in comparison to around 30% for the G-10 membrane. Although the reported MWCO of the G-10 membrane is significantly higher than for the HL membrane, the average permeate flux for the G-10 membrane was significantly lower in comparison to the HL membrane. In general the average permeate flux was affected by the pH and NaCl addition for

both feed sources and both membranes. Both pH and NaCl addition had a significant impact on the total peptide transmisson and organic content transmission but differed according to feed source and membrane type. Nanofiltration is an interesting and promising method for the separation of peptides.

As an analysis method of the different peptides in the feed sources and filtration fractions, RP-HPLC was used. Yeast extract contains more hydrophilic compounds than hydrophobic compounds. Primatone on the other hand was found to have a more hydrophobically balanced peptide distribution. A PCA plot also confirmed the differences between the two feed sources.

The peak heights obtained by the derivatization of the amino acids and peptides of each sample were compared by principal component analysis (PCA) showing several interesting aspects. Although the molecular weight of the different peptides could not be determined, different peptides could be separated. The PCA analysis showed a different peptide distribution between the ultrafiltration fractions and the nanofiltration fractions. For Primatone filtered with the HL membrane, the permeate containing salt were different from the fractions without salt. Furthermore the permeate fractions of yeast extract separated with the G-10 membrane at pH 8 resulted in a different sample composition than at pH 4.

According to the differences observed during the nanofiltration and the RP-HPLC analysis, peptide fractionation was achieved. Nanofiltration is a possible method for the fractionation of peptides.

The bioactivity of the nanofiltration fractions was tested as a nutrient additive to a serum-free media in CHO cells and showed interesting preliminary results. The productivity is not always related to the cell density, as the highest overall specific β–interferon productivity was achieved for low cell density conditions similar to the hydrolysate free negative control. Furthermore, the separation of yeast extract with the G-10 membrane at pH 8, displayed the highest cell density after a 4 day period and was twice as high as the positive control containing 0.025% crude yeast extract. Moreover, the cell density was higher than the positive

control containing 0.2% Primatone. Further analysis and testing is required to improve reproducibility and stability.

Overall the fractionation of protein hydrolysates by nanofiltration is a promising method for the enrichment of protein hydrolysates with bioactivity and should be investigated in more detail. The preliminary results obtained from the testing of the bioactivity showed that the fractionation of peptide mixtures by nanofiltration could be a useful tool for the development of a supplement for a serum-free media for CHO cells.

9.2 Recommendations

The studies described in this thesis focused on the identification of the operating conditions which significantly affects performance during a nanofiltration fractionation of peptide mixtures and the characterization of the fractions and their assessment as a potential supplement for serum-free media in a CHO cell line. Nanofiltration fractionation was significantly affected by membrane type, feed composition, pH, and salt addition and this was elucidated through statistical methods. Future work and recommendations include the following studies which can be divided in two parts; the characterization of the peptides and the further investigation of the nanofiltration operation.

1) Characterization of peptides

- Growth and productivity enhancement testing in CHO cells with the nanofiltration fractions with added NaCl after a desalting step.
- A total fingerprint of the fractionation samples by HPLC-MS or MALDI-TOF to investigate the molecular weight of the different peptides present in the feed, permeate and retentate fractions.
- Using the obtained peptide fraction fingerprints the isoelectric point of each peptide could be calculated according to the amino acid sequence and would provide the charge of the peptides (positive, neutral, or negative). Knowing the charge of the separated peptides would help in understanding and predicting the selectivity of a membrane for peptide transmission.

- Isolation of single peptides, with subsequent testing of the isolated peptides stimulating affect on CHO cell culture growth and production rate

Analysis and isolation of peptides would help in the development of an additive for a serum-free media. The investigation would be labor and cost intensive, but would lead to an understanding of the importance of each peptide and its stimulating factor. Combinations of different peptides may also help in growth stimulation and should be tested.

2) Nanofiltration operation

- Investigation of the influence of the feed concentration, transmembrane pressure, temperature, and crossflow velocity on filtration performance
- Investigation and comparison of additional membrane types
- Comparison of different yeast extract batches on the filtration performance

Carrying out the recommendations in part 2 would provide more information about the filtration performance of a nanofiltration fractionation of peptide mixtures. The application of nanofiltration for peptide fractionation is a new approach and not yet completely understood. It depends greatly on both the membrane and peptide properties.

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Appendices

Appendix A: Abbreviations

A	Cross section area
ACE	Angiotensin converting enzyme
Ca^{2+}	Calcium
CHO	Chinese hamster Ovary cells
Da	Dalton
EGF	Epidermal growth factor
ELISA	Enzyme linked immunosorbent assay
EPO	Erythropoietin
bFGF	Fibroblast growth factor
FCR	Folin & Ciocalteu's phenol reagent
FDA	Food and drug administration
IGF	Insulin-like grwoth Factor
IPP	Isoleucin-proline-proline
J	Permeate flux
kDa	Kilo dalton
HL-60	Human promyelocytic leukemia-60 cell line
HPLC	High performance liquid chromatography
L	Length
LC	Liquid chromatography
LMH	Litres per square meter per hour
MALDI	Matrix assisted laser desorption ionization
M-CSF	Macrophage-colony stimulating factor
Mg^{2+}	Magnesium
MPa	Megapascal
MS	Mass spectrometry
MWCO	Molecular weight cut-off
n.a.	Not available

NZCase	Casein hydrolysate
NaCl	Sodium Chloride
OPA	o-phthaldialdehyde
PDGF	platelet-derived growth factor
PITC	Phenylisothiocyanate
PSTI	Pancreatic secretory trypsin inhibitor
R	Resistance
RP	Reverse phase
SBTI	Soy bean trypsin inhibitor
SPTI	Serine palmitoyltransferase inhibitor
SEC	Size exclusion chromatography
SeO_3^{2-}	Selenium trioxide
Sf-9	Spodoptera frugiperda-9 cell line
TEA	Triethylamine
TFA	Trifluoroacetic acid
TOF	Time of flight
TSHR	Thyroid stimulating hormone receptors
UV	Ultraviolet
VCR	Volume concentration ratio
VPP	Valine-proline-proline
Zn^{2+}	Zinc
ε	Dielectric constant of electrolyte
ε_0	Vacuum permittivity
η	Viscosity
δ	Zeta potential

Appendix B: Additional Plots for Membrane Characterization Chapter

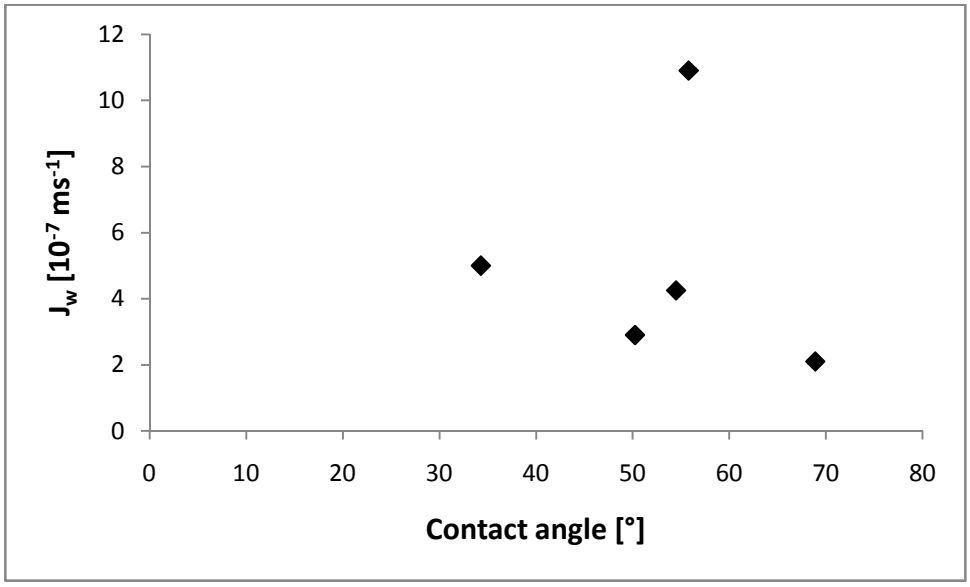

Figure B-1 Relationship between contact angle versus water flux

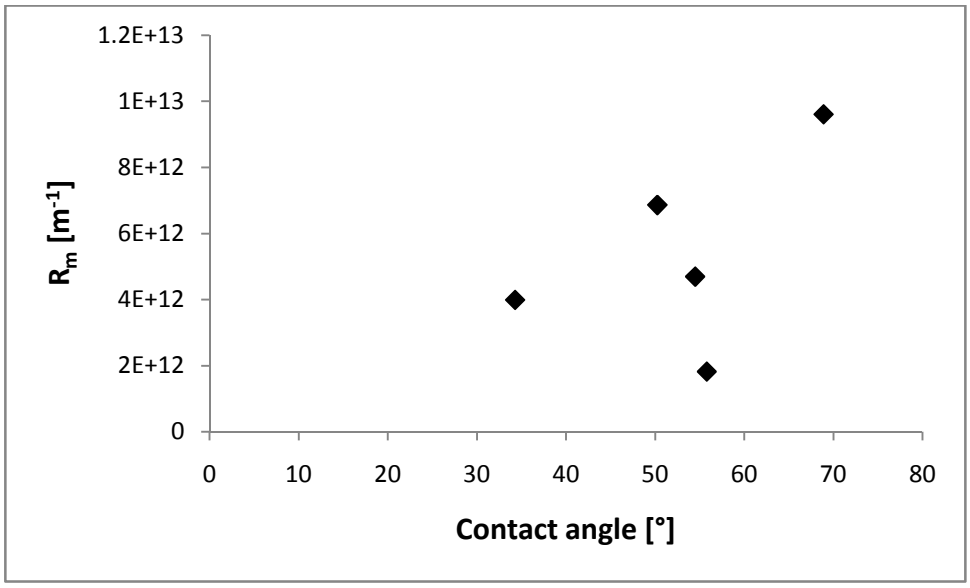

Figure B-2 Relationship between contact angle versus membrane resistance

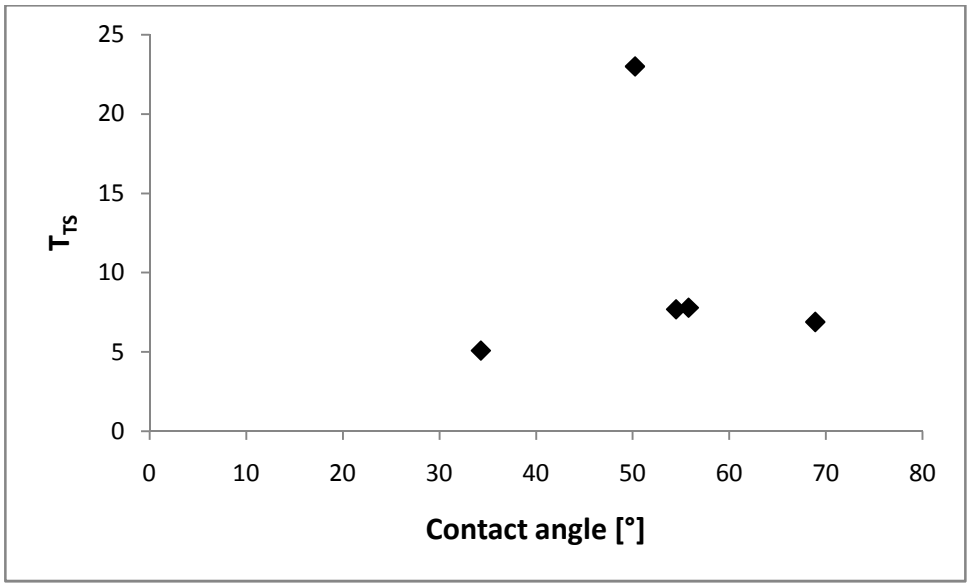

Figure B-3 Relationship between contact angle versus transmission of total solids

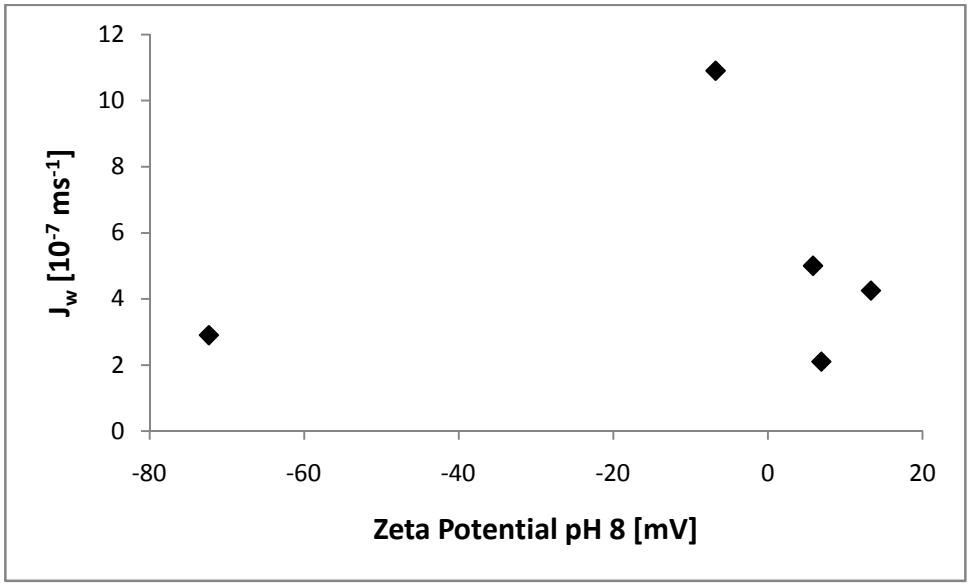

Figure B-4 Relationship between zeta potential versus water flux

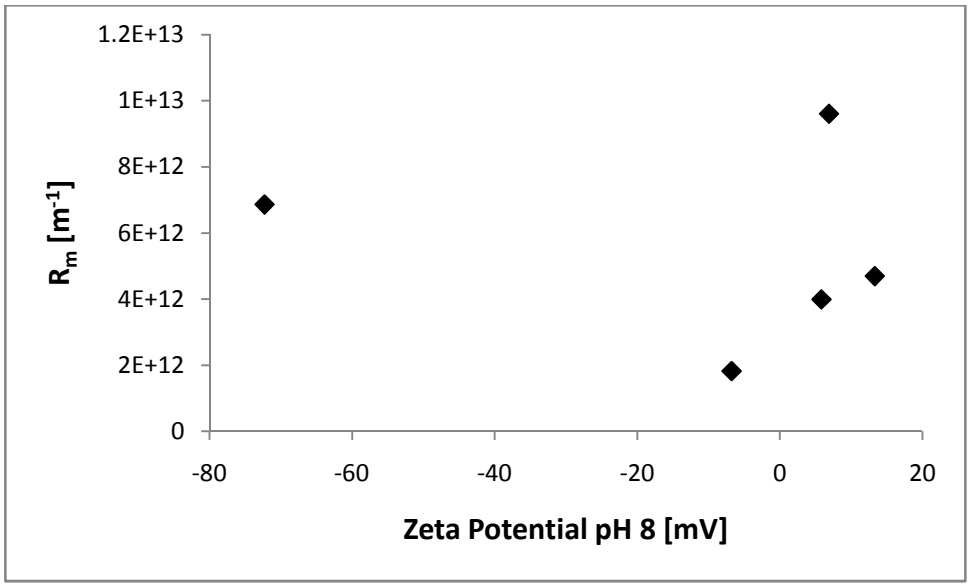

Figure B-5 Relationship between zeta potential versus membrane resistance

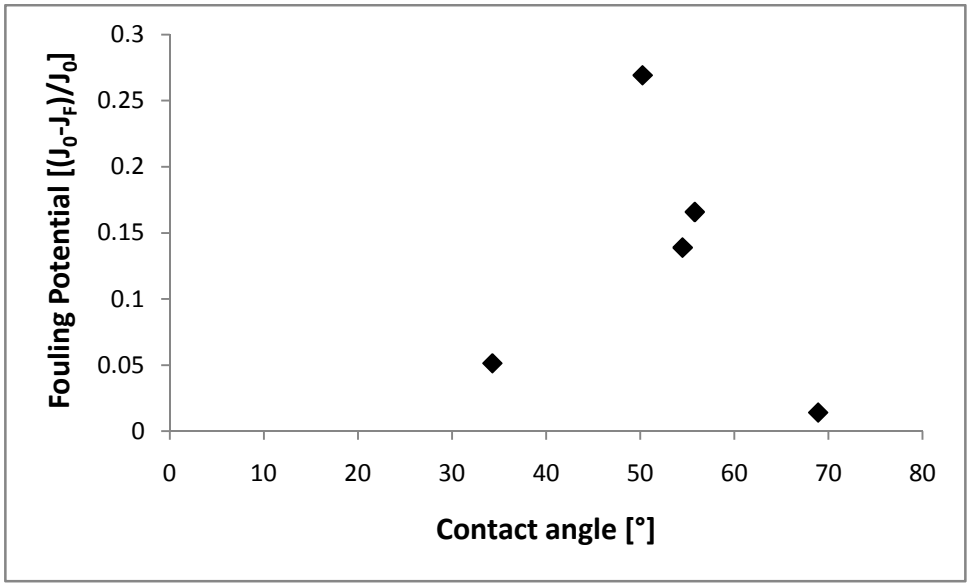

Figure B-6 Relationship between contact angle and fouling potential

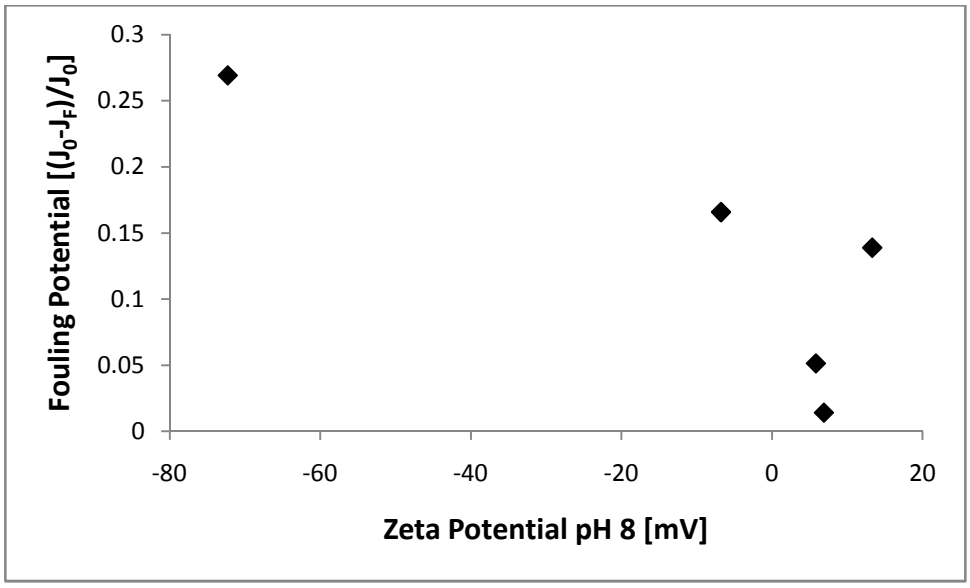

Figure B-7 Relationship between zeta potential and fouling potential

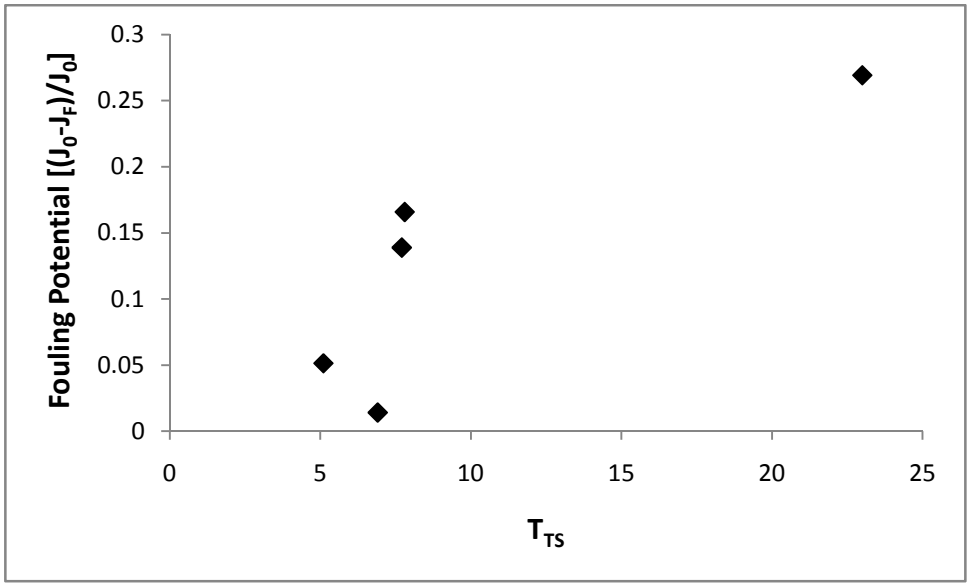

Figure B-8 Relationship between transmission of total solids and fouling potential

Appendix C: Raw data for PCA analysis

					Retention times [min]				
Sample	**Membrane**	**Feed**	**Fraction**	**Condition**	**1.66**	**1.91**	**2.21**	**2.4**	**2.64**
1	HL	YE	Retentate	pH 4 +	0.0	1024.5	103.1	0.0	374.5
2	HL	YE	Retentate	pH 4	0.0	907.5	98.1	0.0	369.0
3	HL	YE	Retentate	pH 8 +	0.0	981.0	96.1	0.0	355.0
4	HL	YE	Retentate	pH 8	0.0	783.5	75.6	0.0	266.5
5	HL	Primatone	Retentate	pH 4 +	0.0	156.1	44.7	0.0	88.0
6	HL	Primatone	Retentate	pH 4	0.0	415.0	111.5	0.0	227.5
7	HL	Primatone	Retentate	pH 8 +	0.0	260.0	65.7	0.0	142.9
8	HL	Primatone	Retentate	pH 8	0.0	323.5	85.9	0.0	172.0
9	G-10	YE	Retentate	pH 4 +	0.0	206.0	24.2	0.0	62.2
10	G-10	YE	Retentate	pH 4	0.0	563.5	76.7	0.0	184.0
11	G-10	YE	Retentate	pH 8 +	0.0	357.5	49.9	0.0	96.3
12	G-10	YE	Retentate	pH 8	0.0	615.5	497.0	0.0	126.5
13	G-10	Primatone	Retentate	pH 4 +	0.0	89.0	23.3	0.0	45.6
14	G-10	Primatone	Retentate	pH 4	0.0	288.5	78.0	0.0	161.5
15	G-10	Primatone	Retentate	pH 8 +	0.0	303.5	71.8	0.0	137.5
16	G-10	Primatone	Retentate	pH 8	0.0	293.5	71.8	0.0	131.0
17	HL	YE	Permeate	pH 4 +	0.0	789.5	0.0	0.0	509.5
18	HL	YE	Permeate	pH 4	0.0	992.0	0.0	0.0	580.5
19	HL	YE	Permeate	pH 8 +	14.9	229.0	184.5	0.0	135.5
20	HL	YE	Permeate	pH 8	0.0	462.5	33.1	0.0	365.5
21	HL	Primatone	Permeate	pH 4 +	14.3	82.3	44.9	32.1	70.2
22	HL	Primatone	Permeate	pH 4	54.9	356.5	180.0	130.5	272.0
23	HL	Primatone	Permeate	pH 8 +	12.9	114.5	96.3	60.2	154.0
24	HL	Primatone	Permeate	pH 8	0.0	217.5	194.0	0.0	381.5
25	G-10	YE	Permeate	pH 4 +	7.4	218.0	0.0	0.0	117.7
26	G-10	YE	Permeate	pH 4	13.2	721.5	0.0	0.0	349.0
27	G-10	YE	Permeate	pH 8 +	7.2	190.5	0.0	0.0	247.5
28	G-10	YE	Permeate	pH 8	7.3	245.5	0.0	0.0	313.5
29	G-10	Primatone	Permeate	pH 4 +	0.0	169.5	47.9	0.0	120.6
30	G-10	Primatone	Permeate	pH 4	12.7	265.5	95.1	0.0	204.5
31	G-10	Primatone	Permeate	pH 8 +	0.0	133.7	51.6	0.0	188.0
32	G-10	Primatone	Permeate	pH 8	0.0	83.8	59.1	0.0	224.5
33	10kDa	Primatone	Feed	UF	35.4	327.4	81.2	0.0	179.2
34	10kDa	Primatone	Retentate	UF	28.9	252.1	64.4	0.0	136.5
35	10kDa	Primatone	Permeate	UF	35.1	342.8	83.9	0.0	191.1
36	10kDa	YE	Feed	UF	75.8	708.8	0.0	0.0	276.9
37	10kDa	YE	Retentate	UF	68.9	569.4	75.1	0.0	212.0
38	10kDa	YE	Permeate	UF	54.3	710.7	0.0	0.0	256.6
	Retention times [min]								

Sample	2.91	3.15	3.5	4.1	4.8	5.59	7.2	7.95	9.64
1	305.5	0.0	167.0	491.0	0.0	53.2	6.3	15.5	211.5
2	314.5	0.0	173.0	500.0	0.0	51.0	0.0	13.5	209.0
3	293.0	0.0	158.5	460.5	0.0	50.6	0.0	15.4	189.5
4	214.5	0.0	112.5	343.0	0.0	36.3	0.0	11.2	144.5
5	72.2	0.0	41.8	83.4	0.0	24.7	8.5	1.5	61.1
6	187.5	0.0	109.5	204.5	0.0	61.8	21.4	6.4	148.5
7	121.2	0.0	70.0	133.6	0.0	38.6	13.6	5.0	99.0
8	140.5	0.0	84.3	154.5	0.0	45.5	16.7	9.0	115.0
9	48.8	0.0	23.7	85.4	0.0	0.0	0.0	0.0	39.6
10	143.0	0.0	66.7	238.0	0.0	23.3	0.0	5.2	112.8
11	77.7	0.0	36.0	128.0	0.0	12.8	0.0	0.0	60.1
12	240.5	0.0	153.4	208.9	210.5	44.8	6.3	12.2	163.0
13	39.0	0.0	22.5	41.0	0.0	9.4	0.0	0.0	26.7
14	136.0	0.0	83.4	146.5	0.0	40.8	17.4	0.0	98.0
15	123.0	0.0	66.6	130.0	0.0	35.6	19.0	0.0	90.1
16	111.4	0.0	68.4	122.0	0.0	41.2	18.0	0.0	84.3
17	565.5	0.0	208.0	634.0	0.0	55.1	5.9	10.5	197.4
18	697.0	0.0	234.0	763.0	0.0	397.0	0.0	16.0	209.0
19	150.8	154.5	215.2	236.5	248.0	34.7	0.0	5.3	118.1
20	409.0	0.0	131.5	505.5	0.0	35.0	0.0	7.9	151.5
21	80.7	0.0	28.7	63.3	0.0	11.4	0.0	0.0	34.1
22	324.5	0.0	114.5	238.0	0.0	45.2	5.9	10.3	116.5
23	210.0	0.0	71.1	172.5	0.0	38.1	0.0	0.0	98.1
24	411.5	0.0	162.0	338.5	0.0	98.5	15.1	8.1	186.0
25	126.2	0.0	38.3	173.4	0.0	18.2	0.0	0.0	50.7
26	357.0	0.0	110.5	465.0	0.0	52.8	0.0	11.9	165.0
27	257.5	0.0	83.4	365.5	0.0	35.6	0.0	0.0	141.5
28	301.5	0.0	92.2	406.5	0.0	24.9	0.0	5.7	145.5
29	111.3	0.0	55.0	116.9	0.0	32.6	6.2	0.0	71.7
30	190.5	0.0	99.3	194.5	0.0	52.5	18.8	5.1	118.0
31	179.0	0.0	81.4	182.0	0.0	46.6	6.6	0.0	114.6
32	205.0	0.0	103.5	210.5	0.0	31.4	6.3	0.0	128.2
33	155.8	0.0	82.2	156.3	0.0	48.4	14.3	8.7	110.2
34	119.2	0.0	63.0	122.4	0.0	37.5	11.5	7.4	87.7
35	166.7	0.0	88.3	166.1	0.0	50.2	14.6	8.4	115.6
36	225.8	0.0	103.8	320.9	0.0	35.7	11.0	9.7	133.5
37	172.2	0.0	78.0	244.0	0.0	28.0	10.9	10.8	104.3
38	211.3	0.0	97.5	307.5	0.0	32.7	8.6	7.4	13.1

Retention times [min]

Sample	11	11.572	15.57	15.84	20.56	21.2	21.52	21.93	22.2
1	35.9	25.9	253.5	0.0	148.0	36.8	103.9	100.5	173.5
2	44.9	26.3	249.5	0.0	152.0	34.7	84.5	107.3	0.0
3	29.1	23.5	229.0	0.0	139.0	34.6	98.0	91.8	0.0
4	29.5	17.9	189.0	0.0	107.0	28.0	70.2	66.9	0.0
5	19.8	14.7	114.2	0.0	60.7	12.6	23.7	40.8	0.0
6	50.3	35.6	234.0	0.0	141.5	24.0	51.3	87.4	0.0
7	32.4	24.1	171.0	0.0	94.9	17.2	36.2	64.5	0.0
8	36.5	28.0	200.0	0.0	110.1	19.1	38.7	63.3	0.0
9	5.9	0.0	55.8	0.0	30.9	0.0	19.0	34.8	0.0
10	24.9	7.6	148.5	0.0	86.7	22.4	58.4	74.3	0.0
11	14.4	0.0	89.5	0.0	53.7	6.5	32.4	52.4	0.0
12	35.2	18.6	194.0	0.0	117.5	28.3	74.3	90.8	0.0
13	5.4	0.0	51.5	0.0	26.6	0.0	14.3	25.1	0.0
14	32.1	25.5	179.5	0.0	86.8	15.7	35.3	53.6	0.0
15	33.0	24.1	172.0	0.0	81.0	17.7	35.0	55.4	0.0
16	27.1	22.0	156.5	0.0	74.6	16.2	34.5	49.8	0.0
17	43.4	26.6	251.7	0.0	176.4	18.2	82.3	136.2	0.0
18	61.9	34.0	237.0	0.0	202.5	24.6	78.0	153.0	0.0
19	17.8	17.7	159.0	0.0	115.5	12.3	51.0	80.1	0.0
20	40.0	22.2	191.0	0.0	142.0	15.5	60.7	102.2	0.0
21	12.5	6.1	64.8	0.0	44.3	0.0	14.1	34.2	0.0
22	46.2	35.9	208.5	0.0	147.5	0.0	38.0	47.0	34.6
23	34.8	30.2	187.0	0.0	115.5	0.0	35.5	73.9	0.0
24	69.3	58.0	307.0	146.0	217.5	0.0	66.8	154.5	444.5
25	15.9	5.9	66.0	23.8	57.9	0.0	44.0	57.6	0.0
26	42.1	21.2	207.5	0.0	141.5	0.0	104.9	114.8	0.0
27	31.3	15.1	182.0	0.0	107.2	0.0	82.0	100.1	0.0
28	35.4	17.2	184.5	0.0	126.0	0.0	66.2	106.7	0.0
29	23.8	18.1	126.4	0.0	66.0	0.0	28.7	53.4	0.0
30	41.8	33.2	208.0	0.0	113.5	0.0	42.7	78.1	0.0
31	33.8	27.9	191.5	0.0	102.0	0.0	35.7	65.9	0.0
32	37.1	31.7	203.5	0.0	117.2	0.0	26.5	63.3	0.0
33	40.1	31.5	149.7	0.0	90.9	41.0	0.0	74.1	0.0
34	33.7	36.3	130.6	0.0	75.3	32.8	0.0	55.7	0.0
35	42.8	29.3	160.6	0.0	92.2	42.6	0.0	75.6	0.0
36	35.5	18.2	148.9	0.0	97.3	83.5	0.0	83.5	0.0
37	27.8	14.2	119.1	0.0	0.0	66.5	0.0	66.0	0.0
38	34.7	16.9	15.5	0.0	91.8	7.9	0.0	78.6	0.0

	Retention times [min]								
Sample	**22.5**	**23.52**	**23.8**	**24.2**	**24.5**	**24.9**	**25.9**	**26.9**	**29.1**
1	303.0	86.5	0.0	21.3	26.4	23.9	8.6	21.7	0.0
2	292.0	88.1	0.0	21.2	25.9	24.7	26.0	24.9	23.1
3	297.5	80.4	0.0	20.4	24.3	22.9	12.5	20.6	10.6
4	263.5	69.9	0.0	15.6	18.5	17.4	7.0	19.9	6.0
5	192.5	54.9	0.0	24.6	3.5	3.0	9.9	13.6	9.5
6	326.0	69.4	0.0	48.1	13.4	11.1	15.8	15.4	13.7
7	260.5	56.9	0.0	33.9	6.5	5.5	7.5	14.0	6.1
8	285.5	61.3	0.0	37.8	11.5	8.9	12.0	15.0	11.7
9	87.2	46.1	0.0	0.0	0.0	0.0	5.1	15.3	45.6
10	208.5	57.0	0.0	13.2	15.3	14.9	13.6	17.1	5.9
11	128.5	45.6	5.5	5.4	5.9	12.0	10.3	13.5	0.0
12	264.5	63.5	0.0	16.8	19.7	18.5	7.8	19.8	32.2
13	126.0	58.4	0.0	0.0	0.0	0.0	5.1	13.6	5.4
14	279.0	70.8	35.6	0.0	5.1	5.4	14.9	14.7	8.4
15	263.0	60.5	36.2	0.0	11.1	5.4	16.6	13.7	13.7
16	282.0	60.9	32.9	0.0	10.3	5.3	8.6	14.4	7.3
17	217.5	66.7	0.0	8.8	10.7	11.9	26.6	16.1	23.5
18	254.5	75.2	0.0	12.9	15.3	17.8	36.9	21.8	32.7
19	215.5	52.9	0.0	5.2	5.9	10.8	16.7	14.8	16.1
20	228.0	59.8	0.0	6.5	7.3	8.3	26.6	17.7	26.3
21	100.7	47.1	6.9	0.0	0.0	0.0	0.0	13.6	0.0
22	280.0	66.7	29.6	0.0	5.4	11.5	19.0	16.2	16.5
23	275.5	50.3	25.7	0.0	0.0	0.0	16.3	14.5	13.2
24	355.0	70.9	19.7	33.2	15.8	15.2	26.6	16.5	25.8
25	164.1	44.8	8.5	0.0	7.0	7.2	7.4	13.6	37.9
26	290.0	61.6	24.1	0.0	19.4	19.4	23.3	18.7	0.0
27	263.5	49.6	9.7	0.0	13.1	7.7	19.9	14.8	0.0
28	206.5	53.1	19.7	6.3	14.9	14.1	16.8	15.4	39.2
29	246.0	62.0	34.1	0.0	6.1	0.0	6.7	13.5	5.7
30	304.0	73.2	49.3	0.0	13.7	6.3	16.1	14.7	14.3
31	290.5	58.9	36.7	0.0	7.5	0.0	6.8	12.9	6.0
32	233.5	61.6	42.9	0.0	12.8	5.8	13.9	14.1	8.1
33	211.2	24.0	33.9	8.8	9.2	0.0	17.5	6.2	22.2
34	182.2	21.1	27.3	7.2	9.2	0.0	16.7	0.0	16.2
35	230.0	25.7	36.1	9.3	9.7	0.0	20.3	0.0	23.0
36	207.1	33.5	22.5	18.4	18.2	0.0	0.0	0.0	22.9
37	172.7	26.7	18.8	15.3	15.3	0.0	0.0	0.0	24.2
38	211.3	32.8	21.6	17.6	18.0	0.0	0.0	0.0	25.7

	Retention times [min]

Sample	29.4	29.65	30.46	30.5	30.92	33.22	33.9
1	80.5	25.8	49.1	0.0	36.1	119.6	0.0
2	108.0	27.0	48.6	0.0	35.4	131.6	0.0
3	101.5	28.5	52.3	0.0	35.4	238.5	0.0
4	84.0	25.3	49.2	0.0	35.2	136.0	0.0
5	61.0	20.4	36.9	0.0	36.8	290.5	0.0
6	75.6	20.6	35.1	0.0	37.0	295.5	0.0
7	65.8	21.4	36.7	0.0	37.0	276.5	0.0
8	69.9	10.3	37.2	0.0	37.0	318.0	0.0
9	46.1	8.5	94.5	0.0	42.0	916.5	640.0
10	63.4	19.7	45.3	0.0	38.7	1014.0	0.0
11	69.0	19.0	36.3	0.0	39.0	282.5	0.0
12	44.4	10.4	65.7	0.0	54.6	1437.0	0.0
13	105.0	27.1	49.9	0.0	34.9	267.5	0.0
14	88.7	11.4	44.1	0.0	35.0	286.0	0.0
15	89.3	25.5	44.7	0.0	34.9	256.0	0.0
16	73.5	12.5	46.5	0.0	35.3	340.0	0.0
17	109.7	11.7	39.5	0.0	35.2	197.0	0.0
18	177.5	14.0	99.2	22.3	35.5	215.0	0.0
19	59.2	24.1	41.5	0.0	35.3	160.1	0.0
20	96.2	12.6	49.1	0.0	35.3	87.1	0.0
21	71.4	21.6	39.0	0.0	37.3	265.5	0.0
22	93.0	23.1	39.0	0.0	37.5	331.5	101.0
23	63.3	21.1	37.2	0.0	37.3	229.0	0.0
24	88.6	0.0	35.7	18.7	163.9	266.0	0.0
25	38.8	10.6	38.6	0.0	38.8	277.0	0.0
26	73.4	0.0	39.2	0.0	39.5	858.5	0.0
27	88.4	10.6	39.7	0.0	39.3	255.0	0.0
28	51.9	9.8	43.1	0.0	39.0	750.0	0.0
29	70.5	25.5	50.6	0.0	35.3	420.0	107.0
30	89.6	25.4	45.2	0.0	35.3	300.0	0.0
31	65.8	25.0	44.8	0.0	35.0	256.0	0.0
32	71.2	23.0	42.3	0.0	34.9	299.5	0.0
33	23.5	0.0	13.6	0.0	20.4	53.3	0.0
34	26.6	0.0	12.4	0.0	18.5	22.2	0.0
35	26.0	0.0	13.7	0.0	22.4	52.1	0.0
36	31.5	0.0	16.1	0.0	22.7	26.3	0.0
37	19.4	0.0	12.3	0.0	19.6	57.9	0.0
38	28.2	0.0	17.9	0.0	25.5	48.4	0.0

(All peaks presented in mAU)

Appendix D: Loading plots for PCA analysis

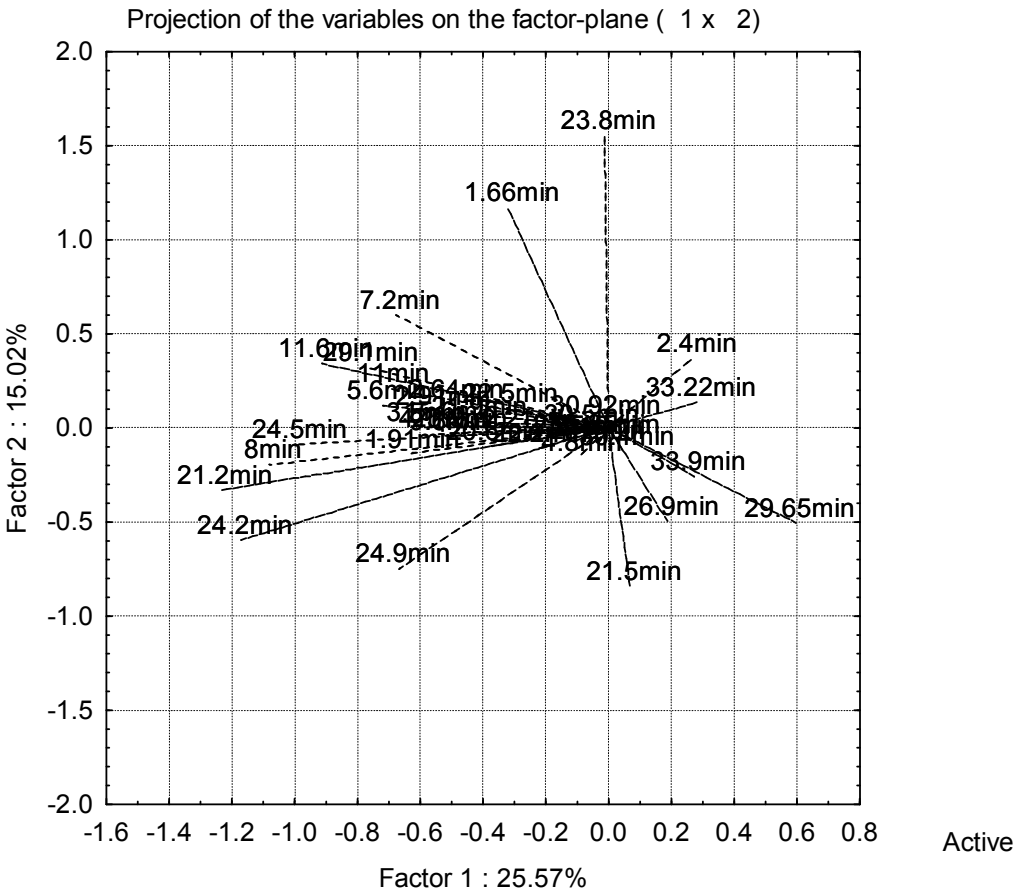

Figure D-1 Loading plot for PCA plot used with the overall design (all fractions)

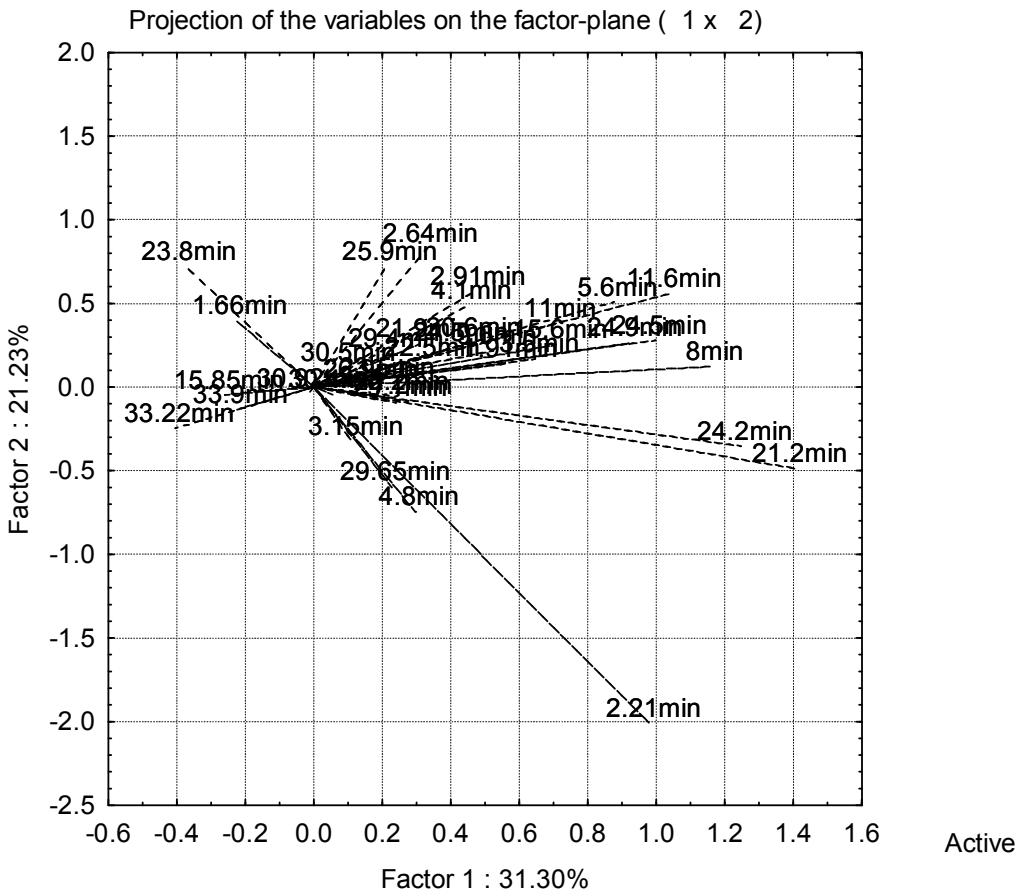

Figure D-2 Loading plot for PCA plot used with the yeast extract nanofiltration fractions obtained with both membranes

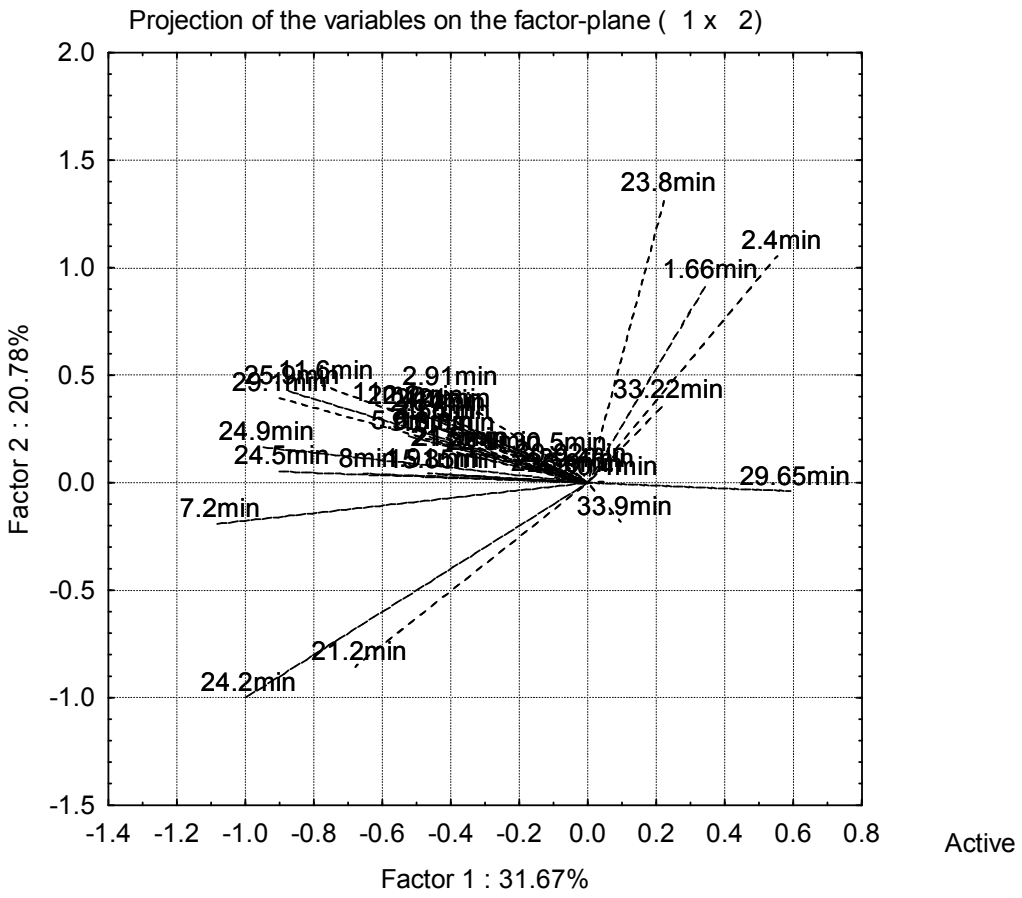

Figure D-3 Loading plot for PCA plot used with the Primatone nanofiltration fractions obtained with both membranes

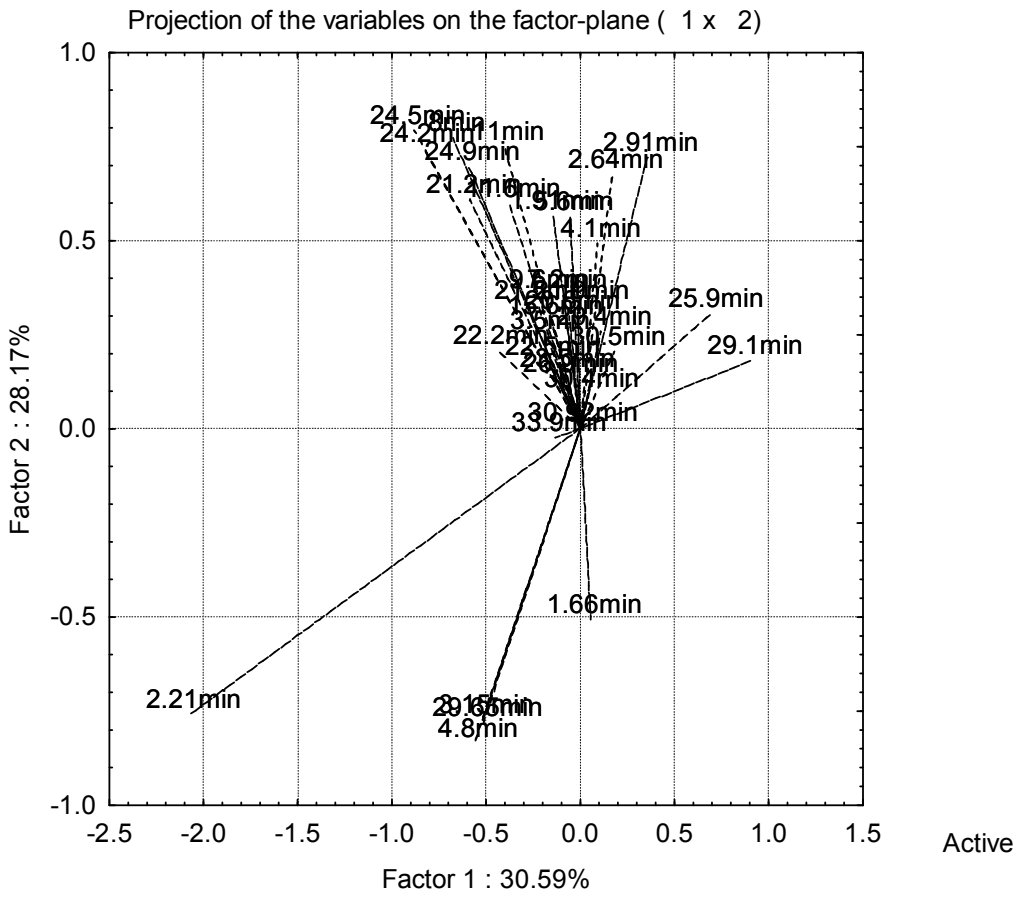

Figure D-4 Loading plot for PCA plot used with the factorial design 1 (yeast extract and HL membrane)

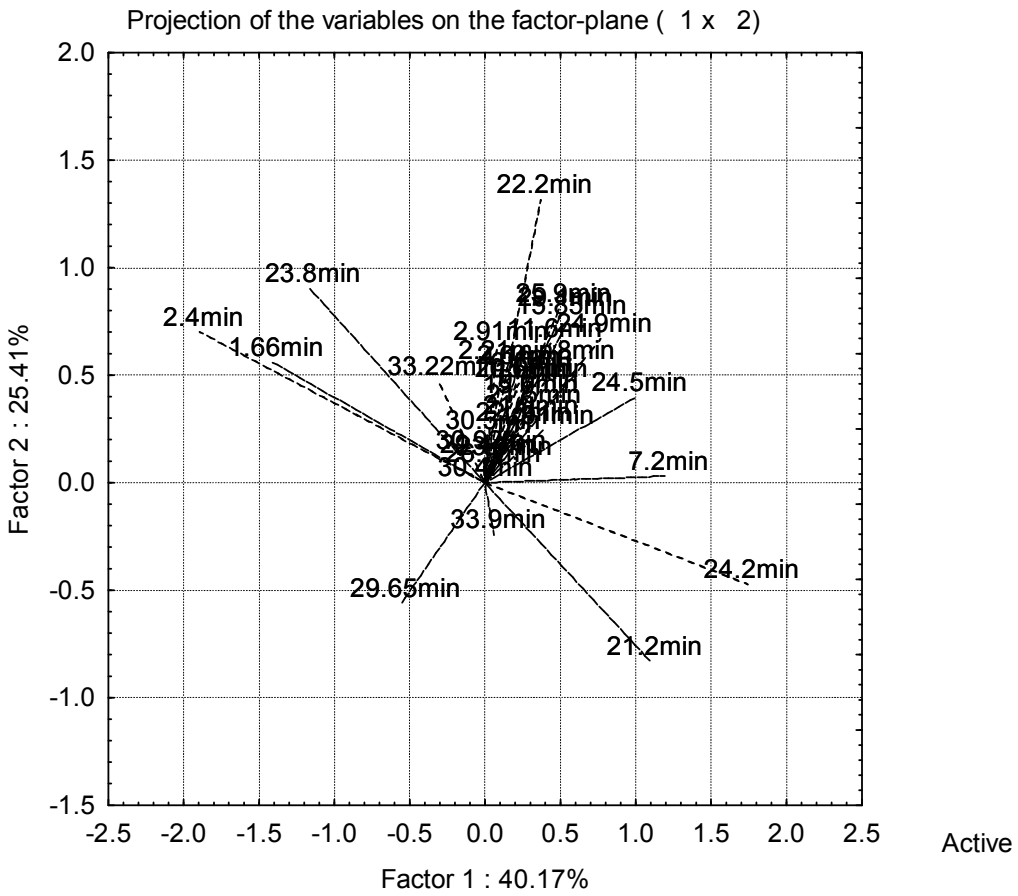

Figure D-5 Loading plot for PCA plot used with the factorial design 2 (Primatone and HL membrane)

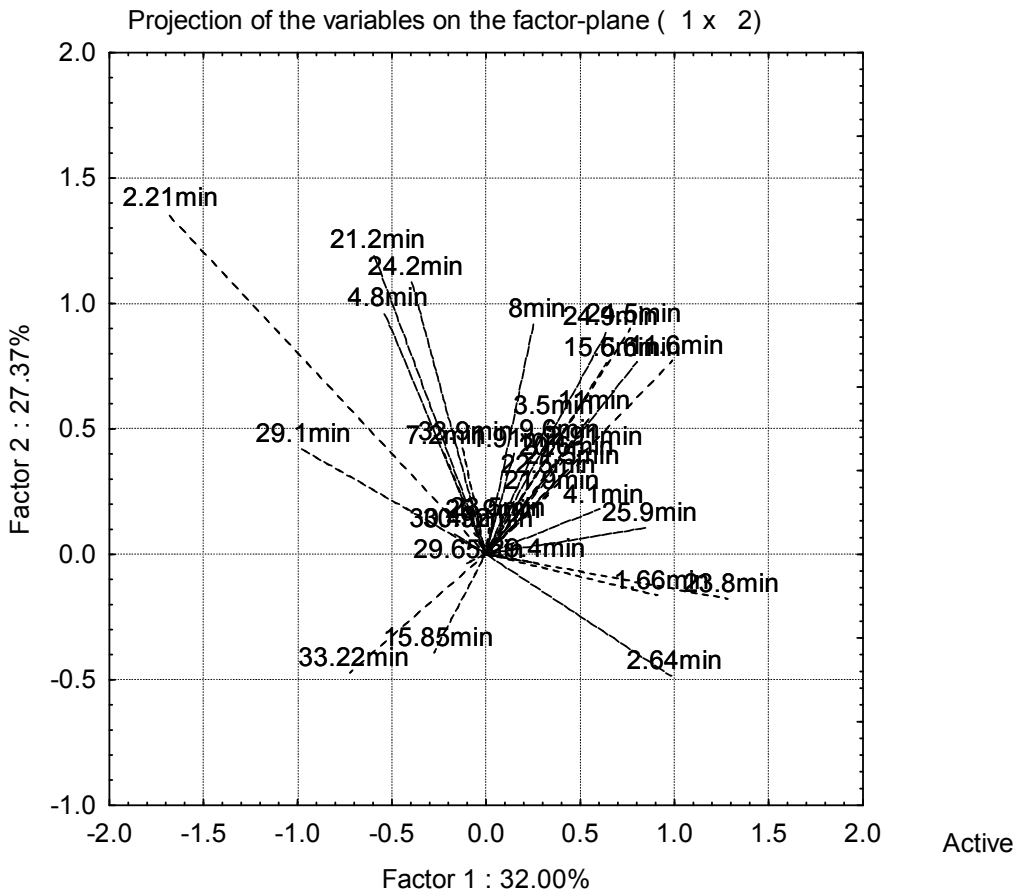

Figure D-6 Loading plot for PCA plot used with the factorial design 3 (yeast extract and G-10 membrane)

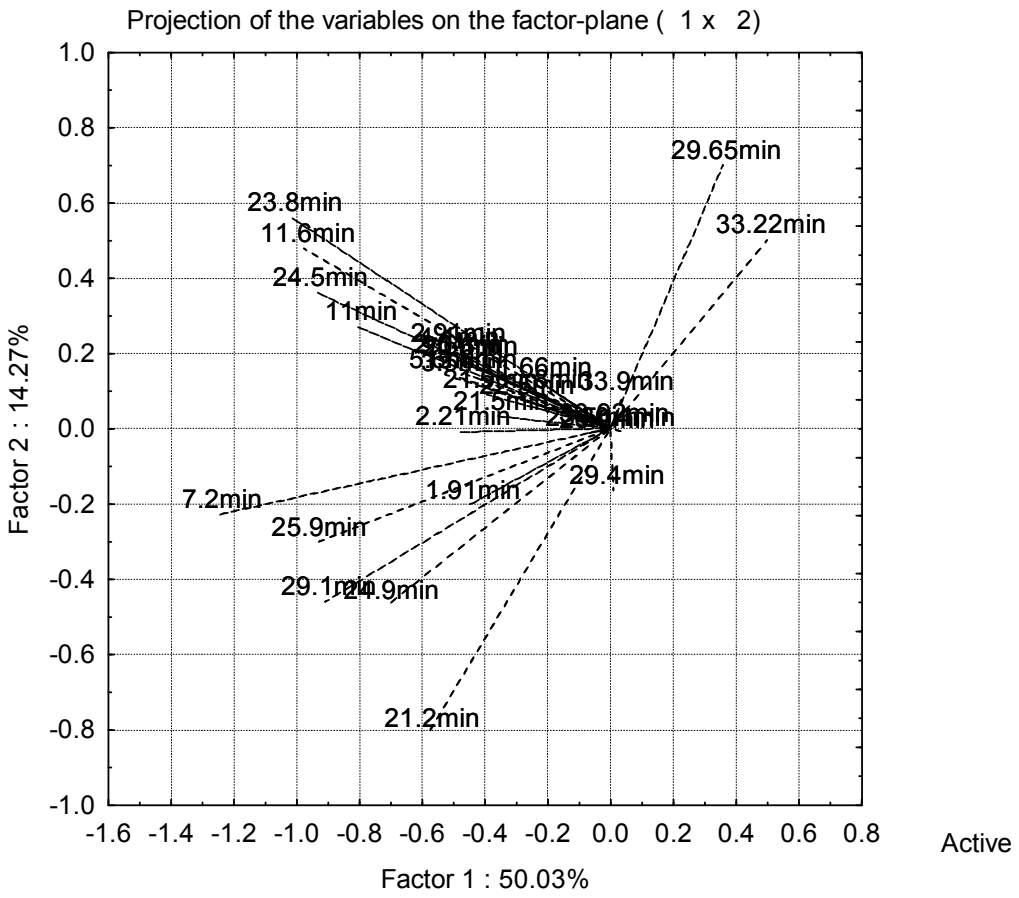

Figure D-7 Loading plot for PCA plot used with the factorial design 4 (Primatone and G-10 membrane)

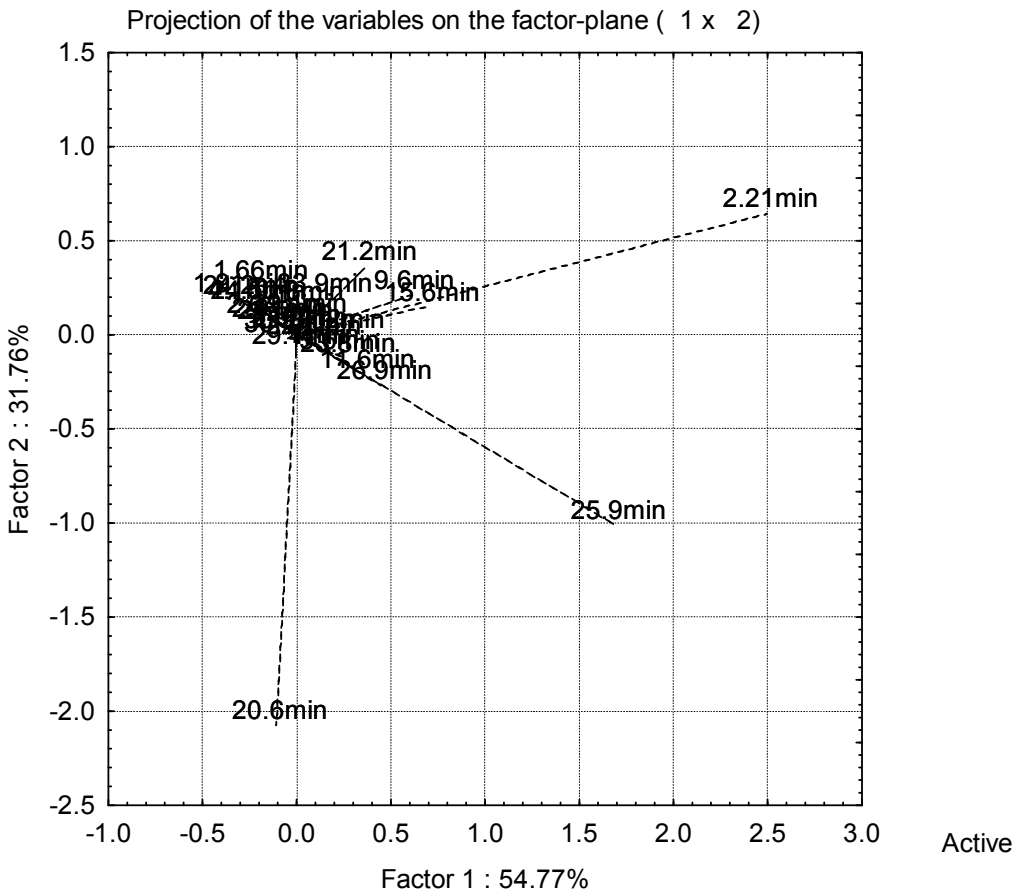

Figure D-8 Loading plot for PCA plot used with the ultrafiltration fractions